CANADIAN THESES ON MICROFICHE

THÈSES CANADIENNES SUR MICROFICHE

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

Canada

NL 339 (r. 86/09)

0-315-21212-8

16

National Library of Canada
Canadian Theses Division
Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Division des thèses canadiennes

# PERMISSION TO MICROFILM — AUTORISATION DE MICROFILMER

- Please print or type — Écrire en lettres moulées ou dactylographier

| | |
|---|---|
| Full Name of Author — Nom complet de l'auteur<br>Anita Yee Mei Ma | |
| Date of Birth — Date de naissance<br>July 13, 1959 | Country of Birth — Lieu de naissance<br>Hong Kong |
| Permanent Address — Résidence fixe<br>10 B, Hoover Court 218 - 220 Argyle St.<br>Kowloon, Hong Kong | |
| Title of Thesis — Titre de la thèse<br>The Use of Protease in the Production of Sauce from Canola Meal | |
| University — Université<br>University of Alberta | |
| Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée<br>MSc in Food Processing | |
| Year this degree conferred — Année d'obtention de ce grade<br>1985 | Name of Supervisor — Nom du directeur de thèse<br>B. Ooraikul |

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

| | |
|---|---|
| Date<br>January 18/85 | Signature<br>Anita Yee Mei Ma. |

NL-91 (4/77)

THE UNIVERSITY OF ALBERTA

# The Use of Protease in the Production of Sauce from Canola Meal

by

Anita Yee Mei Ma

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF
Master of Science
in
Food Processing

Department of Food Science

EDMONTON, ALBERTA
Spring 1985

# THE UNIVERSITY OF ALBERTA

## RELEASE FORM

NAME OF AUTHOR Anita Yee Mei Ma

TITLE OF THESIS The Use of Protease in the Production of Sauce from Canola Meal

DEGREE FOR WHICH THESIS WAS PRESENTED Master of Science

YEAR THIS DEGREE GRANTED Spring 1985

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(SIGNED) Anita Yee Mei Ma

PERMANENT ADDRESS:

10B Hoover Court
218 - 220 Argyle St.
Kowloon Hong Kong

DATED January 18 1985

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled The Use of Protease in the Production of Sauce from Canola Meal submitted by Anita Yee Mei Ma in partial fulfilment of the requirements for the degree of Master of Science in Food Processing.

Supervisor

Robert T. Hardin

Monica Palcic

Peter Sporns

Date January 4 1985

To the Lord of the Universe,
with love

# Abstract

Canola meal was prehydrolyzed with Alcalase 0.6L, a proteolytic enzyme, in order to expedite subsequent sauce fermentation. Conditions employed for the hydrolysis were optimized by means of response surface methodology and were found to be: temperature 69 C, pH 9, enzyme-substrate ratio 0.31 (v/w), meal-solvent ratio 1:5 (w/v), and reaction time 2 h. The hydrolyzate was then subjected to koji preparation with two different mold cultures, viz., *Aspergillus oryzae* and *Aspergillus sojae*. Subsequently, moromi was prepared by mixing 18% brine solution with the koji, and was left to ferment for 5 weeks.

The canola sauce produced was then analyzed for total nitrogen (TSN), amino nitrogen (AN), amino acids, total titratable acidity (TTA), organic acids, reducing sugars, total sugars, salt and color. The results were: 1.12-1.34% (w/w) TSN, 0.66-0.80% (w/v) AN, 16.65-29.95 mg/100 mL TTA, 7.43-9.67% (w/v) reducing sugars, 8.69-15.39% (w/v) total sugars, 18.72-20.37% (w/v) salt. These results were quite comparable to those in Kikkoman shoyu, a Japanese soy sauce.

All 16 amino acids found in canola sauce appeared to be slightly lower in concentration than those found in commercial soy sauce. Among the two most important amino acids, concentration of glutamic acid in canola sauce was 61.8-87.8 mMoles/mL as compared to 86.6 mMoles/mL in the commercial sauce, while that of aspartic acid was 24.2-39.5 mMoles/mL versus 65.7 mMoles/mL.

Nine organic acids were identified. Concentrations of most of the acids in canola sauce were quite similar to those in commercial sauce, except lactic and propionic acids which were significantly lower in canola sauce. This was attributed to the deficiency in lactic acid fermentation in the moromi stage. Levulinic acid, commonly found in chemical soy sauce and responsible for the undesirable aroma was not detected in canola sauce prepared in this experiment.

Color of canola sauce was found to be more yellowish while that of Kikkoman shoyu was more reddish.

Sensory evaluation showed canola sauce samples to be comparable to Chinese and synthetic soy sauce in their acceptability. However, they were rated inferior to Kikkoman shoyu, because of the general lack of traditonal soy sauce aromas.

# Acknowledgements

The author wishes to express her deepest appreciation to her supervisor Dr. Ooraikul for his encouragement and support during this research. His patience and critical review of this thesis are invaluable.

Special thanks are extended to Dr. Sporns, Dr.Hardin and Dr. Hadziyev for their advice throughout the course of this work.

Sincere gratitude and appreciation are due to Dr. Palcic for her serving as a committee member.

Thanks are extended to Margaret Miko for her assistance in amino acid analysis.

Special thanks are due to all her fellow colleaques, especially to Gour Choudhury and Bill Howe, for their friendship and assistance in compiling this manuscript.

Thanks are also extended to Western Canadian Seed Processor Ltd. for their supply of the canola meal.

Financial support from AART, which made completion of this project possible, is gratefully acknowledged.

## Table of Contents

Chapter Page

1. INTRODUCTION ....................................1

2. LITERATURE REVIEW ...............................5

2.1 Soy Sauce ......................................5

2.1.1 History ......................................5

2.1.2 Ingredients ..................................6

2.1.3 Types and Grades .............................6

2.1.4 Trade in Soy Sauce ...........................8

2.2 Manufacturing Process ..........................10

2.2.1 Definitions of Terminologies .................10

2.2.2 Conventional Approach ........................11

2.2.2.1 Traditional manufacturing practices ........11

2.2.2.2 Current modifications ......................12

2.2.3 Chemical Approach ............................16

2.2.4 Semi-chemical Approach .......................18

2.2.5 Comparison of the Three Approaches ...........18

2.3 Biochemical and Microbial Changes in Koji and Moromi ....21

2.3.1 Enzymes ......................................21

2.3.1.1 Proteases ..................................21

2.3.1.2 Carbohydrases ..............................26

2.3.1.3 Others enzymes .............................30

2.3.2 Changes Taking Place in Koji and Moromi ...31

2.3.3 Microbiology in Moromi Mash ..................35

2.4 Chemical Composition of Soy Sauce ..............42

2.4.1 Nitrogen Containing Compounds ................42

2.4.2 Carbohydrates ..............................47
2.4.3 Acids and Related Compounds ..............48
2.4.4 Principle Flavor Components ..............52
2.4.5 Color ...................................55
2.5 Alternative Materials and Processes for the Sauce Production ..............................58
2.5.1 Use of Other Substrates ..................58
2.5.2 Preliminary Study Using Canola Meal as a Raw Material .............................59
2.5.2.1 Results of the study .............60
2.5.2.2 Problems encountered .............64
3. EXPERIMENTAL ....................................67
3.1 Optimization of total soluble nitrogen in Canola Meal-enzyme Hydrolyzate ................67
3.1.1 Experimental Design ......................67
3.1.1.1 One-factor-at-a-time approach .....68
3.1.1.2 Central composite rotatable design ..............................70
3.1.2 Enzymatic Hydrolysis ......................71
3.1.2.1 Materials: .......................71
3.1.2.2 Equipment ........................74
3.1.2.3 Procedure ........................74
3.2 Production of Koji .............................75
3.2.1 Rehydration of Freeze-dried Mold Culture ..75
3.2.1.1 Materials ........................75
3.2.1.2 Equipment ........................75
3.2.1.3 Procedure ........................75
3.2.2 Koji Preparation ..........................76
3.2.2.1 Materials ........................76

3.2.2.2 Equipment ..........................77

3.2.2.3 Procedure ..........................77

3.3 Moromi Fermentation ................................78

3.4 Extraction and Pasteurization ....................79

3.4.1 Equipment ..................................79

3.4.2 Procedure ..................................79

3.5 Analysis of Canola Sauce .........................81

3.5.1 Fractionation of Soluble Components .......81

3.5.1.1 Materials and equipment ...........81

3.5.1.2 Procedure ..........................82

3.5.2 Analysis of Nitrogen Containing Compounds .85

3.5.2.1 Quantitation of amino nitrogen ....85

3.5.2.2 Quantitation of total soluble nitrogen ..........................85

3.5.2.3 Analysis of amino acids by means of Automatic Amino Acid Analyzer ..87

3.5.3 Analysis of Acidic Compounds ..............87

3.5.3.1 Quantitation of total titratable acidity ..........................87

3.5.3.2 HPLC Analysis of Organic Acids ....88

Materials ..........................88

Equipment ..........................88

Procedure ..........................89

3.5.4 Analysis of Sugars .........................95

3.5.4.1 Determination of reducing sugar ...95

3.5.4.2 Determination of non-reducing sugar ..............................95

Sample inversion ..................95

Analytical procedure ..............96

3.5.5 Total Soluble Solids Measurements .........96

3.5.6 PH Measurements ..........................96

3.5.7 Sodium Chloride Determination ............96

3.5.8 Color Measurement ........................97

3.5.9 Moisture Content Determination ...........97

3.6 Sensory Evaluation ...........................99

4. RESULTS AND DISCUSSION .......................100

4.1 Optimization of Enzymatic Hydrolysis on Canola-meal ................................100

4.2 Koji and Moromi Preparations ................117

4.3 Changes in total soluble solids and pH during Moromi Fermentation ........................122

4.4 Composition of Canola Sauces ................126

4.4.1 Nitrogen Containing Compounds ...........126

4.4.1.1 Total soluble nitrogen (TSN) and amino nitrogen (AN) ..............126

4.4.1.2 Amino acid profile ..............129

4.4.2 Titratable Acidity and Organic Acid Profile ..................................132

4.4.3 Salt Concentration .......................141

4.4.4 Total and Reducing Sugar Contents ........141

4.4.5 Color .....................................145

4.5 Sensory Evaluation ...........................147

5. CONCLUSIONS ...................................153

6. SUGGESTIONS FOR FURTHER RESEARCH ..............155

7. BIBLIOGRAPHY ...................................157

# List of Figures

| No. | Description | Page |
|---|---|---|
| 1 | Conventional manufacturing of soy sauce | 14 |
| 2 | Production of chemical soy sauce | 17 |
| 3 | Chromatogram of organic acids of fermented and chemical soy sauce | 20 |
| 4 | Protease in koji inoculated with A.oryzae strain 1989 | 24 |
| 5 | Protease in moromi inoculated with *A.oryzae* strain 1989 | 25 |
| 6 | a-amylase in koji and moromi | 27 |
| 7 | b-amylase in koji and moromi | 28 |
| 8 | Reducing sugars in koji inoculated with *A.oryzae* strain 1989 | 32 |
| 9 | Total soluble nitrogen, amino nitrogen abd ammonia nitrogen in koji inoculated with *A.oryzae* strain 1989 | 34 |
| 10 | Reducing sugars in moromi | 36 |
| 11 | Total soluble in moromi | 37 |
| 12 | Viable counts of *L.delbruckii* , *Sacch. rouxii* and pH in soy mash | 39 |
| 13 | Flow chart of conventional fermentation process on canola sauce | 61 |
| 14 | Flow chart of semi-chemical process on canola sauce | 62 |
| 15 | Changes in soluble solids and | |

pHs during the conventional fermentation 63

16 Changes in soluble solids and pHs during the semichemical process 65

17 Flow chart of enzymatic production of canola sauce 80

18 Fractionation of soluble components in canola sauce 84

19 Calibration curve of ninhydrin-leucine color yield 86

20 Calibration curves of standard isocitric acid and glyoxylic acid 90

21 Calibration curves of satandard propionic acid and pyruvic acid 91

22 Calibration curves of standard succinic acid and malic acid 92

23 Calibration curves of standard lactic acid and trans-aconitic acid 93

24 Calibration curves of standard formic acid and citric acid 94

25 Calibration curve of sodium chloride solution 98

26 Effect of temperature on %total soluble nitrogen yields during enzymatic hydrolysis 103

27 Effect of pH on %total soluble nitrogen yields during enzymatic hydrolysis 104

28 Enzymatic hydrolysis on protein
105

29 Effect of meal-solvent ratio on %total soluble nitrogen yield during enzymatic hydrolysis 106

30 Effect of duration of hydrolysis on %total soluble nitrogen yield during enzymatic hydrolysis 107

31 Effect of enzymatic-substrate ratio on %total soluble nitrogen yield during enzymatic hydrolysis 109

32 Three dimensional illustration on optimal condition for enzymatic hydrolysis. PH was fixed at 9.02 118

33 Three dimensional illustration on optimal condition for enzymatic hydrolysis. Temperature was fixed 68.96 C 119

34 Three dimensional illustration on optimal condition for enzymatic hydrolysis on canola meal. Enzyme-substrate ratio was fixed at 0.3878 120

35 Changes in total soluble solids during enzymatic production of canola sauce 123

36 Changes in pH during enzymatic production of canola sauce 125

37 HPLC chromatogram of organic acid profile in canola sauce prepared with *A.oryzae* at pH 5.5 134

38 HPLC chromatogram of organic acid profile in canola sauce prepared with *A.sojae* at pH 5.5 135

39 HPLC chromatogram of organic acid profile in canola sauce prepared with *A.oryzae* & *A.sojae* at pH 5.5 136

40 HPLC chromatogram of organic acid profile in canola sauce prepared with *A.oryzae* at pH 6.5 137

41 HPLC chromatogram of organic acid profile in commercial soy sauce 138

# List of Tables

| No. | Contents | Page |
|---|---|---|
| 1 | Nutrient composition of soybean and rapeseed meals | 3 |
| 2 | Organic acid contents soy sauce | 50 |
| 3 | Codings for each level of the variables | 72 |
| 4 | Central Composite Rotatable Design | 73 |
| 5 | Observed and predicted results percentage total soluble nitrogen | 113 |
| 6 | Results of percentage total soluble nitrogen with coded data | 114 |
| 7 | The coded and uncoded variables of the stationary point | 116 |
| 8 | Results of nitrogen containing compounds | 127 |
| 9 | Contents of amino acids in canola sauce and soy sauce | 130 |
| 10 | Total acidity and organic acid composition and concentration of canola sacue and soy sauce | 134 |
| 11 | Salt contents of the canola sauce and the commercial soy sauce | 142 |
| 12 | Sugar contents of the canola sauce and the commercial soy sauce | 144 |
| 13 | Color measurements of the canola sauce and the commercial soy sauce | 146 |

14 Average sensory evaluation scores of canola sauce and Kikkoman soy sauce 148

15 Average sensory evaluation scores of canola sauce and Chinese soy sauce 149

16 Average sensory evaluation scores of canola sauce and China Lily 151

# 1. INTRODUCTION

The discovery of soy sauce is one of the greatest prides of Oriental food manufacturers. Its production does not only designate a masterpiece of success in food industry, but also carries an appreciation of one of the cultural arts in the orients.

Soy sauce is an all purpose seasoning agent, characterized its distinctive myriad aromas and flavors. It is the most popular sauce in oriental countries. In Japan alone, about 1,400,000 tonnes of soy sauce are produced annually, over 95% of which is consumed locally (Wood, 1982). However, the sauce is gaining acceptance in other parts of the world especially in North America. In Canada, the production of soy sauce is virtually non-existant due to limited market and inadequate supply of soybean. The annual ten million dollar worth of soy sauce market in Canada relies heavily on the importation from Japan, Hong Kong, Taiwan and the U.S.A.. Therefore , the potential for the development of the sauce manufacturing industry in Canada is good as long as the cost and quality is competitive. Cost competition may be achieved by using cheaper raw materials or cheaper production methods. The two main traditional ingredients of the sauce are wheat and soybean. Canada is a major wheat growing country, but not soybean. Therefore, a good locally grown substitute for soybean must be found. Among high protein seeds that may be used for the purpose, rapeseed stands out prominently due to its importance in

Canadian economy and its high protein quality (Sosulski and Sarwar, 1973).

Rapeseed is the major oilseed in Canada. Cultivation of rapeseed has a long history. The earliest direct references to rapeseed were found in India from 2000 to 1500 B.C. (Singh, 1958). The knowledge of rapeseed cultivation spreaded to Europe in the thirteenth century. Commercial growing of this crop in Canada began in 1942. Today, rapeseed ranks the fifth among the major oilseeds of the world. Expansion of the production was found mainly in five areas, viz., Canada, Western Europe, Poland, India and China.

The meal after oil extraction is usually used as aniamal feed or fertilizer. Campbell and Eggum (1980) found that canola (a modern name for Canadian rapeseed) meal had very high protein quality, an average of 90% in Biological Values and 76% in Net Protein Utilization. Summers (1973) showed that canola meal had as high as 40% protein. Table 1 shows the comparison of nutrient composition between rapeseed and soybean meals.

Due to its high protein content and cheaper price, rapeseed meal appears ideal, therefore, as a soybean substitute in the sauce production . Both conventional fermentation and chemical approach using acid hydrolysis have been studied (Ooraikul et al. 1980). However, the conventional fermentation is too lengthy while the chemical method is too corrosive and expensive to be practical for large-scale operation. It is imperative, therefore,

Table 1. Nutrient composition of soybean and rapeseed meals

| oilseed meals | crude protein % | crude fibre % | metabolizable energy, Kcal/g | %Ca | %P | lysine g/16gN | methione g/16gN |
|---|---|---|---|---|---|---|---|
| soybean | 51.5 | 6.7 | 2.55 | 0.3 | 0.7 | 6.47 | 1.28 |
| rapeseed | 39.7 | 13.9 | 1.87 | 0.7 | 1.3 | 5.55 | 1.70 |

:After Summer, 1973.

that a relatively cheap and efficient technique must be found to produce high quality sauce from canola meal to make the product competitive and viable.

Thus, the two pronged objectives of this research were to study techniques whereby the fermentation time might be shortened and high quality sauce might be produced from canola meal instead of soybean. The approach was to pre-hydrolyze raw materials with a suitable proteolytic enzyme to the point where microbial fermentation could be effected and an acceptable sauce produced in one month or less.

# 2. LITERATURE REVIEW

## 2.1 Soy Sauce

### 2.1.1 History

Soy sauce is a dark brown liquid with salty taste and sharp aroma. It occupies a place of honor as an all purpose seasoning agent as well as a table condiment found nearly every in oriental family. It has gained acceptance in many western countries such as United States, Canada and Europe.

Soy sauce is known by different names in different countries of Asia. For example, it is known as chian-yu in China, si-yu in Thailand, shoyu in Japan, tao-yu in Indonesia, tayo in Philippines and ketjap in Malaysia. Piper and Morse (1943) have recorded more than 50 names being used throughout the orient.

The discovery of soy sauce is one of mankind's greatest contributions to food industry. For the first time, it became possible to produce meat-like flavor from plant origin. There was no complete modern review of the origin of soy sauce readily available to the western workers. Perhaps, the earliest record of soy sauce was found in the book of Chau Lai (one of the thirteen Classics of Confucius) written before 1000 B.C. (Groff, 1919), which stated that the king used one hundred and twenty jars of soy sauce for his ceremonial rite. This implied that the Chinese have been using soy sauce for over three thousand years. The introduction and development of soy sauce to Japan was believed to be associated with Buddhism, in which meat was abstained from the diet of the believers. Fermented soy paste, originated from China, was introduced into Japan in the sixth century by Buddhist priests, and gradually transformed into the present Japanese shoyu (Fukushima,1979).

### 2.1.2 Ingredients

The basic ingredients of soy sauce are soybean, wheat, salt and water. Depending on the local practices of the manufacturers, caramel or molasses, previously heated to 180-190 C is added to the sauce to give a desirable sweetish taste, aroma, darker color and greater viscosity of the sauce. Monosodium glutamate (0.3%) is sometimes also introduced to further ameliorate the flavor of the blended sauce (Lockwood,1947 ; Beans-Arcega, 1966). In general, a preservative such as benzoic acid or propyl- or butyl-p-hydroxy benzoate or sodium benzoate is added to the soy sauce. Recently, however, the trend in Japan seems to be toward either aseptic bottling of shoyu without preservatives added, or bottling shoyu which is fortifed with ethanol as a preservative (Yokotsuka, 1981).

### 2.1.3 Types and Grades

According to Fukushima (1981), there are five different types of soy sauce available in Japan, viz., koikuchi, usukuchi, tamari, shiro and saishikoni. In koikuchi, usukuchi or saishikoni shoyu, the defatted soybeans are mixed with equal amount of roasted wheat and then inoculated with a pure culture starter. In tamari shoyu, however, the proportion of wheat to soybean must be less than 10% while in shiro shoyu, a very high ratio of wheat to soybeans must be used. Koikuichi shoyu is an all purpose seasoning agent with strong aroma, myriad flavor and a deep brownish color. This may be associated with the high percentage of wheat which supplies carbohydrates leading to the production of sugar, alcohol, and organic acids in the final product. Wheat also serves as a source of lignin and glycosides (precusors of vanillic compounds), and provides glutamic acids, hence enhancing the flavor of the sauce (Steinkraus, 1983).

Usukuchi shoyu is characterized by a lighter brown color and milder flavor. It is mainly used for cooking when one wishes to preserve the original color and flavor of the foodstuff.

Tamari or chinese type soy sauce is distinguished from the other Japanese shoyu by its distinctive taste, thick consistency and deep dark color. The poor aroma of the Chinese type soy sauce is ascribed to the lower percentage of wheat used and also to the fact that the soy sauce is unpasteurized. However, this type of soy sauce is vastly used in mainland China and Hong Kong. The remaining two types of soy sauces are produced and consumed only in isolated localities or for special use in Japan.

It is generally recognized by the Japanese Government that there are three grades of soy sauce, that is, special, upper and standard. The grades are differentiated by organoleptic evaluation, contents of total nitrogen, soluble solids other than sodium chloride and color. Special grade is assigned only to soy sauce produced by the traditional fermentation (Fukushima,1979).

The quality and price of shoyu are determined by the nitrogen yield, the total soluble nitrogen and the ratio of amino nitrogen to total soluble nitrogen in the sauce. Nitrogen yield is defined as the percentage of nitrogen of raw material converted to soluble nitrogen, which indicates the efficiency of the enzymatic conversion during the sauce preparation. A ratio of higher than 50% of amino nitrogen to total soluble nitrogen is also an evidence of quality (Hesseltine and Wang, 1979).

### 2.1.4 Trade in Soy Sauce

There are more than 2900 soy brewers in Japan where 47% is made up by family enterprises. Usually less than ten workers are employed in each factory, hence the productivity is very low. Five big enterprises, including Kikkoman dominate the soy sauce industry in Japan. The combined share of these five enterprises makes up about 50% of the total production, while the remaining 50% is shared almost equally by 30 medium size factories and almost 2900 small business

(Wantanabe, 1984).

The successful penetration of the soy sauce market into Europe and North American countries is due to the very positive marketing policies of the manufacturers in Japan, Singapore, Hong Kong and Korea. Wood (1982) has done a very thorough statistical report on the worldwide soy sauce trade. According to his report, Hong Kong, the Korean Republic, Singapore and Japan serve as the four main soy sauce suppliers to a total of 97 countries in 1978. Japan produced 1,434,000 tonnes of soy sauce (calculated with specific gravity of 1.2), with less than 0.5% exported. This implied an annual local consumption of 12.81 Kg of shoyu per head or a daily consumption of 35.1 g of shoyu. Hong Kong exported almost the same amount of soy sauce as that of Japan and had her biggest market in North America especially in the United States. Although Korean Republic and Singapore, exported lesser amounts of soy sauce as compared to the above two countries, they did serve as important suppliers to Near and Middle East, as well as Far Eastern and western Pacific countries respectively.

The statistics also revealed that there was a general trend of increase in the demand of soy sauce. This might be due to the increasing popularity of Chinese cuisine in different parts of the world.

Perhaps, the greatest enigma is the very home of soy sauce, mainland China. Unfortunately, no trade figures are directly available. However, from 1976 to 1978, Hong Kong imported about 13,000 tonnes of soy sauce annually, of which, China's share was over 90% of the total import which was approximately twice Hong Kong's exports to the other countries. Furthermore, of the 1,700 tonnes soy sauce imported in Singapore, about half was from mainland China. The amount of imported soy sauce was approximately equivalent to her total export. These figures hinted at an extensive Chinese trade in this product. Recently, China has opened her door to foreign trades, and it is believed that soy sauce exports will definitely become one of her most important transactions.

## 2.2 Manufacturing Process

### 2.2.1 Definitions of Terminologies

Koji - A yeast or other starter prepared from rice or other materials inoculated with the spores of a mold. For example,*Aspergillus oryzae*. The quoted phrase ' a yeast or other starter .' can be better understood as a fermentation enzyme donor. (A synopsis of Japanese Traditional Fermented Foodstuffs )

Meal - The product resulting from grinding the material remaining after the removal of part of the oil by pressure or solvents from oil seeds; its use is generally restricted to feeds for livestock and poultry.

Moromi - Brine-koji mash usually used in fermentation or production of indigenous fermented foods.

### 2.2.2 Conventional Approach

#### 2.2.2.1 Traditional manufacturing practices

Fermentation of soy sauce was at one time a secret household art. Little information about the mystery of fermentation could be acquired. Most soy sauce was manufactured in small-scale factories which blindly followed the traditional methods passed from father to son. In the past, tremendous efforts with endless amount of time as well as primitive equipment were involved in the manufacture.

Soybean was first boiled until soft in a big semispherical iron pan. After boiling, it was poured into bamboo baskets and mixed with wheat flour manually. The resulting mixture was molded in a dark room, specially designed to have its door faced to the north for light and ventilation control, for about one to two weeks depending on the weather conditions. Usually the manufacturers would stop the molding procedure between November and February, as the weather during this period was unfavorable to produce the mold. Care had to be taken that only the yellow mold was allowed to grow on beans, black mold was removed from the batch whenever found. Following successful molding of the bean-wheat flour mix was the addition of brine. Usually a small volume of brine of very high concentration was added. The whole mash was then put in jars and exposed under the sun for two to six months. This sunning process resulted in an evaporation of the liquid in the jars; and three days before the drawing off of the soy, salt solution was added to replace the evaporated liquid. After the end of the brine fermentation, the liquid accumulated at the bottom of the jars was drawn by siphon into another earthenware jar. This liquid was again allowed to sun for several weeks. The first grade soy sauce was made from this liquid. The second drawing soy or lower quality soy was made by pouring another portion of brine into the remaining residues left in the jars after the first drawing. The jars were again allowed to sun from one to two months. Salt water was again added three days before the drawing, and then sauce was drawn off and sold for lower price (Groff 1919; Fukushima, 1981).

### 2.2.2.2 Current modifications

Sauce prepared by the old traditional method was found to vary in quality because of the contamination of molds and bacteria due to inadequate hygienic standard. Nowadays, soy brewers still follow the concept of the conventional approach. However, with scientific progress and improvement, more sophisticated methods have been introduced in recent years. These allow better control of the fermentation condition during the sauce making and ameliorated quality of the soy sauce produced. New equipment such as continuous pressure cookers, automatic innoculators, automatic mixers, ventilation devices of large perforated vats with forced-air blowing from the bottom of the vats, temperature controls and mechanical devices for turning the substrates during the incubation. The process flowchart of the conventional approach is show in Figure 1.

Soybean or defatted soybean grits are moistened and cooked under steam pressure. This procedure greatly influences the enzymatic digestibility of soybean protein (Yokotsuka, 1981). Kawano (1938) found that greater production of amino acid content in shoyu was directly related to enzymatic digestibility of the cooked soybean. Furthermore, cooking soybeans at a high temperature within a short period of time tended to improve the enzymatic digestibility of the soy protein (Yasuda et al., 1973 a, b; Harada et al., 1966). According to the above finding, soybeans are practicably cooked at 1 Kg/cm for 1 hour in most of the soy manufacturing factories.

Ground roasted wheat is then mixed with the cooked soybean in ratios according to types of sauce intended to be produced. The mixture is inoculated with a small amount of seed mold or a pure culture of *Aspergillus oryzae* or *Aspergillus sojae*. The whole mixture, known as koji, is kept in a large shallow tank with a perforated stainless plate false bottom. Temperature and moisture controlled air is blown from the false bottom in order to ventilate the koji as well as to maintain the temperature at around 30 C. After 48 - 72 h of incubation, the moisture of the mash drops from 40 - 45% to around 25 - 30%. During that time, the koji mass gives off a mild, sweetish musty odor together with a

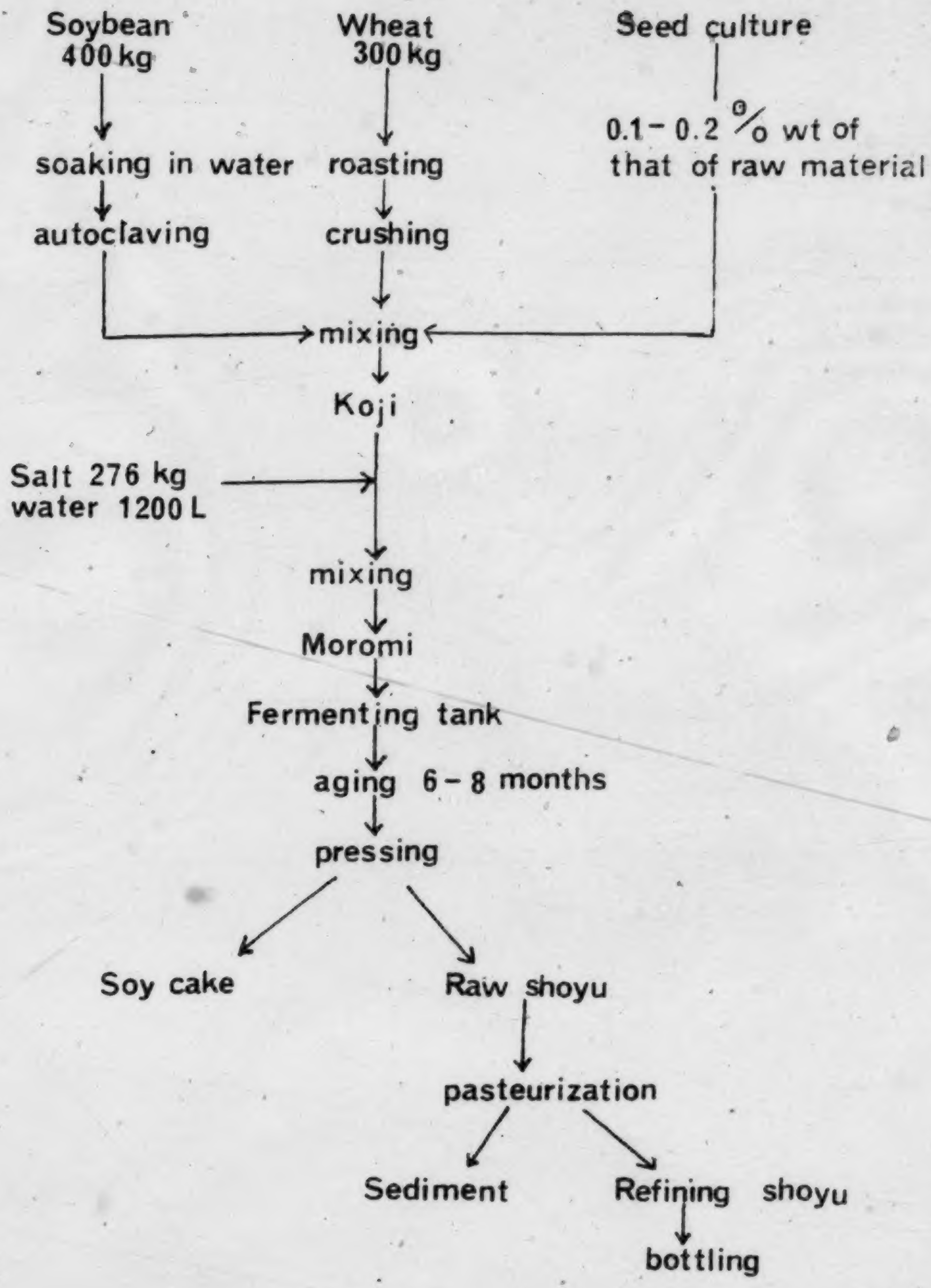

Soybean
400 kg
soaking in water
autoclaving
Wheat
300 kg
roasting
crushing
Seed culture
0.1 - 0.2 % wt of that of raw material
mixing
Koji
Salt 276 kg
water 1200 L
mixing
Moromi
Fermenting tank
aging 6 - 8 months
pressing
Soy cake
Raw shoyu
pasteurization
Sediment
Refining shoyu
bottling

Figure 1 Conventional manufacturing of soy sauce
After Yokotsuka, 1981

greenish yellow mycelium covering the entire mash.

Part of the art of koji preparation relies on the judgement of when to terminate the process. Immature koji results in insuffient hydrolysis of proteins and polysaccharides in the substrates, whereas, delayed termination permits sporulation to occur which will result in the development of unacceptable moldly flavor as well as a sharp, harsh ammonia smell, giving rise to a product of poor quality.

To terminate the koji stage, 18% (w/v) brine is mixed with the koji at the ratio of 1.2 - 1.3 : 1 (v/w). The mash is now called moromi. This denotes the onset of the brine fermentation. Pure culture *Pedioccus halophilus* or *Lactobacillus delbruckii* and *Saccharomyces rouxii* are added at the start and after one month, respectively, to the moromi in order to sustain the fermentation. PH of the moromi drops from the initial value of 6.5 - 7.0 down to 4.7 - 4.9 with the color and flavor becoming more intense. Traditional fermentation continues for one to three years at ambient temperature.

The aged moromi is put into a cloth bag and the liquid part of the mash is separated from the residue with a hydraulic press. The ' raw shoyu ' is then heated to 70 - 80 C for 30 minutes by a plate heater. This heating is necessary to stop the microbial and enzymatic reactions, sediment the heat-coagulated proteins, clarify the shoyu, as well as increase acidity and color of the sauce . A recent tendency toward aseptic bottling of koikuchi type soy sauce in Japan negates the use of preservatives on the product.

### 2.2.3 Chemical Approach

As time and labor becomes costly, a faster and cheaper method of producing soy sauce has to be developed. One of these is a chemical process. Chemical shoyu is prepared by acid hydrolysis of soybean and wheat. The mixture is refluxed with 20% hydrochloric acid for about 12 - 16 h until a maximum concentration of amino nitrogen has been obtained. The hydrolyzate is subsequently neutralized with 50% sodium hydroxide to pH 4 - 5. The sauce is then ready to be placed in hardwood storage tanks for aging prior to bottling. A final salt concentration of the sauce will be 18 - 20% (w/v) if the process is properly carried out (Minor,1945). Figure 2 illustrates the flow-chart of the chemical process.

A milder acid hydrolysis using 18% hydrochloric acid for 8 - 10 h, followed by sodium carbonate neutralization has also been attempted by Watanabe (1969).

Acid hydrolysis usually results in a more complete breakdown of substrates; however, it cannot provide the same kind of flavors as the microbial fermentation process does. In fact, the acid hydrolysed product is more like a solution of amino acids and salt which lacks the subtle aromas and flavors of the fermented soy sauce. Consequently, chemical shoyu has a very limited market in Asia and is often used as

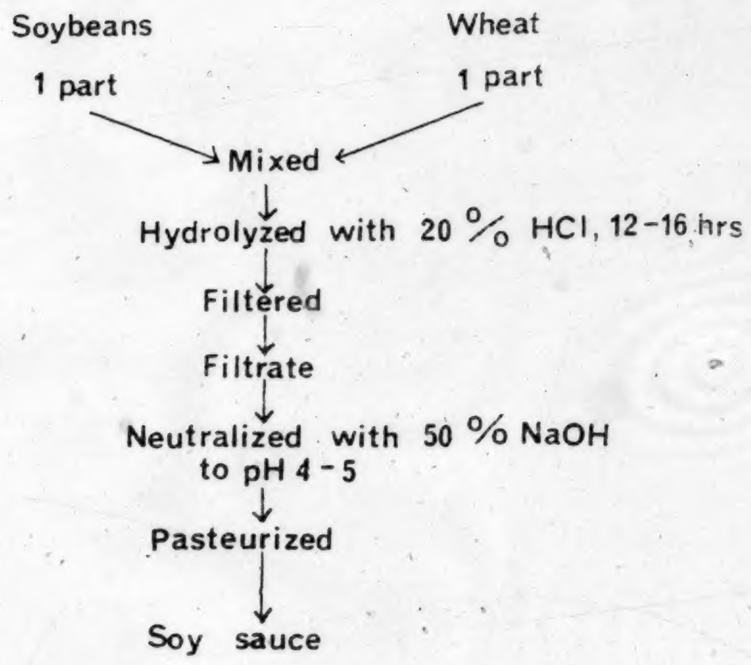

Soybeans
1 part
Wheat
1 part
Mixed
Hydrolyzed with 20 % HCl, 12-16 hrs
Filtered
Filtrate
Neutralized with 50 % NaOH to pH 4-5
Pasteurized
Soy sauce

Figure 2 Production of chemical soy sauce
After Yong and Wood, 1974

an extender of fermented sauce. Moreover, Japanese Agricultural Standard (JAS) regulations state that soy sauce blended with the chemically produced sauce cannot be assigned special grade.

### 2.2.4 Semi-chemical Approach

In order to improve the taste of the chemical soy sauce, an attempt of combining both methods - chemical and fermentative, has been made. Noda Shoyu Co. Ltd. pioneered this semi-chemical approach for large scale production just after the World War II (Yokotsuka, 1960).

In this method, defatted soybeans are partially hydrolyzed with 8 - 10% hydrochloric acid then neutralized with sodium hydroxide. Prehydrolyzed soybeans are subsequently fermented with yeast in the presence of wheat koji. With this method, the fermentation is shortened to 2 - 3 months.

Semi-chemical soy sauce possesses some fermented soy sauce flavors but is essentially a chemical soy sauce, hence still has the similar unpleasant odors as those found in chemical soy sauce. Semi-chemical soy sauce is also called 'shinshiki' in Japan.

### 2.2.5 Comparison of the Three Approaches

Good soy sauce, like good wine, improves its organoleptic characterestics upon storage. The conventional fermentation process is no doubt the best method for producing excellent flavors and aromas. However, with the increasing cost of time, space and labor, the lengthiness of the process becomes a serious disadvantage. The chemical and semi-chemical processes offer a cost advantage over the conventional preparation. Nevertheless, during acid hydrolysis, various secondary reactions occur and produce undesirable compounds such as dark humins, levulinic acid and formic acid which are not present in traditionally fermented soy sauce. Dimethyl sulfide, furfurol and hydrogen sulfide which have very strong objectionably bad odors are derived from methionine, pentose and sulfur-containing amino acids ,respectively, during the chemical hydrolysis. Fukushima (1981) reported that the main organic acid found in chemical soy sauce was formic acid, whereas, the main organic acid in fermented soy sauce was lactic acid, probably produced by lactic acid bacteria during the fermentation. Levulinic acid, not found in traditionally fermented soy sauce, was found in chemical soy sauce (Figure 3). Tryptophan, an essential amino acid, is completely destroyed during the acid hydrolysis.

According to Arcega (1966), yeast converted sugar into alcohol while lactic acid bacteria transformed sugar into lactic acid and other organic acids during fermentation. Upon ripening, alcohol became esterified with the acids, giving the distinctive aroma to the sauce.

In 1977, statistics in Japan indicated that 63% of the bottled soy sauce was composed of fermented product, 30% was

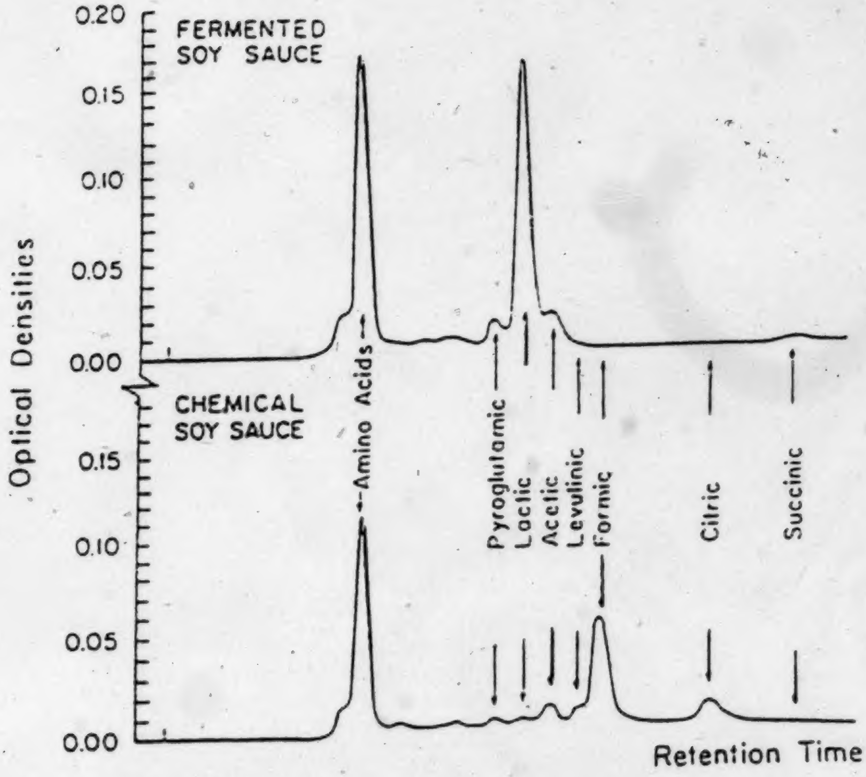


Figure 3 Chromatogram of organic acids of fermented and chemical soy sauce

After Fukushima, 1979

a blended mixture of semi-chemical and fermented sauce, while only 7% was chemical soy sauce (Fukushima, 1982). Semichemical and chemical soy sauce are seldom used alone as table condiments. However, in the western countries, people are less accustomed to fermented sauce products, and therefore would accept chemical soy sauce more readily.

### 2.3 Biochemical and Microbial Changes in Koji and Moromi

### 2.3.1 Enzymes

*Aspergillus oryzae* and *Aspergillus sojae* are often considered as enzyme donors. Various extracellular enzymes such as sucrase, amylases, cellulase, lactase, protease, lipase and phosphatase, etc. are produced, especially during the sporulation of the mold in koji (Yong and Wood, 1977 a,b; Goel and Wood, 1978; Oba, 1974; Kuninaka et al. 1980). According to their functions, three kinds of enzymes, viz., carbohydrases, protease, lipase and other minor enzymes will be discussed. Since most of the enzymes reach the optimal activity level 50 - 70 h after they are produced, in commercial practice, soy sauce makers traditionally permit the koji to develop for 72 h.

### 2.3.1.1 Proteases

The extent of protein hydrolysis is acknowledged as one of the most important factors governing the quality of soy sauce. Soluble low molecular weight peptides and amino acids, which contribute to the palate fullness of soy sauce, are produced through the enzymatic breakdown of proteins in the substrates. Yokotsuka (1977) reported that about 80 - 90% of the protein in the raw materials for soy sauce was solubilized.

Selection of mold strains used for koji making is generally based on the enzymatic activities of the extracellular proteases liberated by the mold. Among the different mold strains used, *A. oryzae* and *A. sojae* were found best for the digestion of soybean protein to produce good quality soy sauce (Nakadai and Nasuno, 1976). Attempts have been made to isolate and characterize proteases produced by the molds. Kundu and Manna (1975) purifed one neutral and one alkaline proteases from *A. oryzae*. The optium pHs for casein and hemoglobin hydrolysis were 6.5 at 60 C and 10.0 at 45 C respectively. Two kinds of neutral proteases, which exhibit a typical endo-proteolytic activity, were also isolated from *A. sojae* (Sekine,1976). Neutral and alkaline proteases differ in their activities in that neutral protease is specific for hydrophobic amino acid residues with the amino side of the bond being broken, whereas alkaline protease is specific for the hydrolysis of the carboxyl side of the aromatic and hydrophobic amino acid residues. Besides neutral and alkaline proteases, the presence of two acid proteases from Takadiastase, a crude powder preparation from *A. oryzae*, were also reported (Tsujia and Endo, 1971). These two proteases were found to have similar amino acid profiles and molecular weight in their protein portion except one of them was a glycoprotein. Both enzymes had pH optima around 3 to 4 and were specific for aromatic or hydrophobic amino acids, capable of cleaving the acids from either end.

It is generally recognized that the total proteolytic activity of koji is well correlated with its neutral and alkaline protease activities. Furthermore, activities of the alkaline and neutral proteases separated from *A. flavus* dropped by approximately 85 to 90% in the presence of 18% sodium chloride medium (Impoolsup et al., 1981). Therefore it seems reasonable to surmise that acid proteases are the chief source of proteolysis in the moromi stage where the pH of the mash is at around 4.8 - 5.0 and that the neutral and alkaline proteases dominate in the koji stage of soy sauce production.

Protease activity in koji was detected from the start of the mold inoculation. A rapid rise of the activity was found between twentieth and thirtieth hour of incubation, with the optimal activity found around the fiftieth hour, followed by a slight decline and fluctuation in activity level for the rest of the koji stage (Figure 4 , Yong and Wood, 1977a). Upon addition of salt in the moromi stage, there was a sharp decrease in protease activity during the first week of fermentation, followed by a complete recovery and then a decline in the activity as the fermentation proceeded (Figure 5, Yong and Wood, 1977b). The initial decrease of protease level right after the salt addition

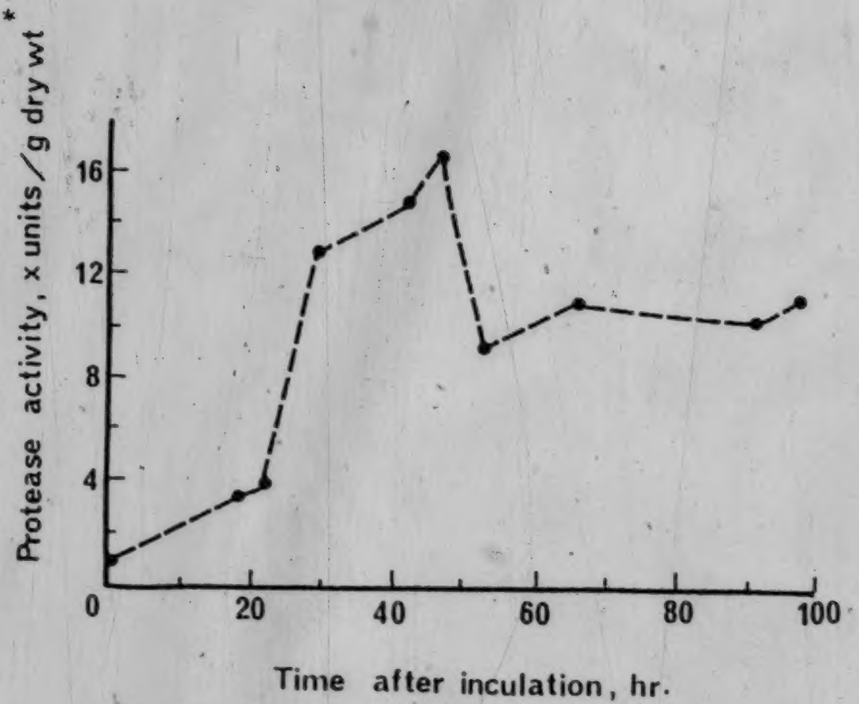


Figure 4 Protease in Koji inoculated with A. oryzae strain 1989. After Yong and Wood. 1977a

*Protease X unit is defined as an enzyme, a solution of which containing 1,5 g per litre, which under the stated experimental condition produced a filtrate with an optical density of 0.500 when measured in a 10 mm path length cell, had a strength of 36 X units per gram.

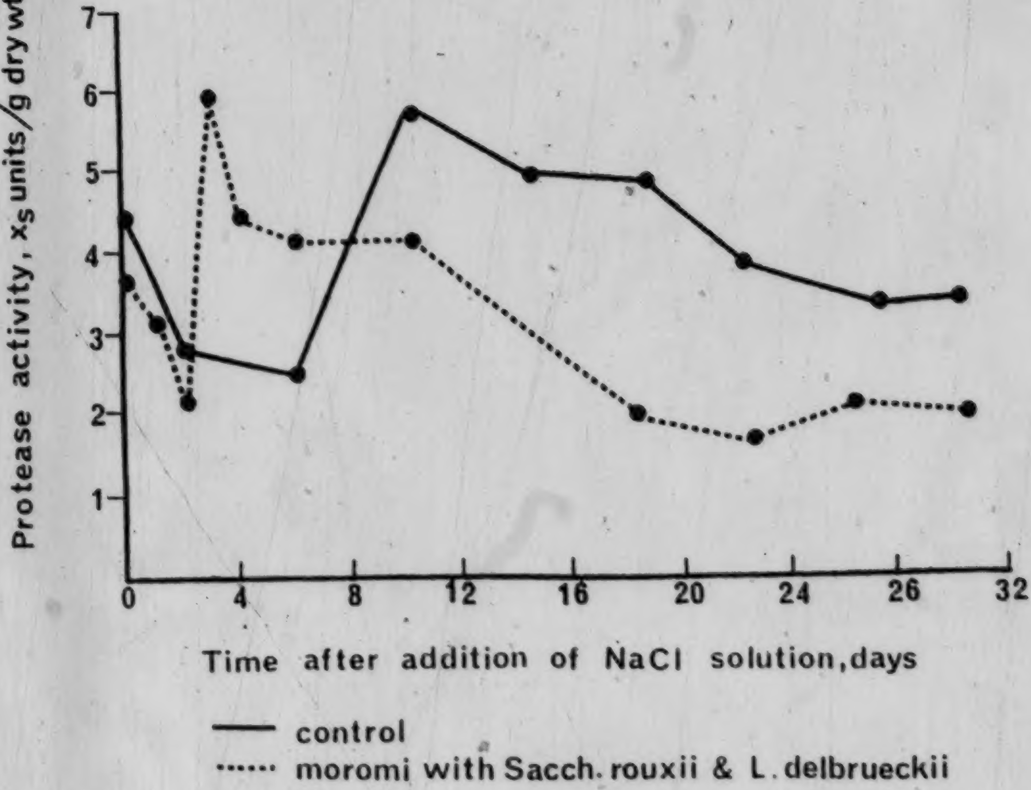


Figure 5 Protease in moromi
S After Yong and Wood, 1976b

may be due to the denaturation or precipitation of the enzyme, followed by re-solubilization and/or release of fresh enzyme upon lysis of mold hyphae.

### 2.3.1.2 Carbohydrases

A considerable number of sugar enzymes have been isolated from the extracellular hydrolyzases present in koji. These include α- and β-amylases, sucrase, cellulase, amyloglucosidase, maltase, α-galactosidase and lactase. Disaccharides and oligosaccharides of the raw materials are broken down by these enzymes with a consequent increase in reducing sugar content, especially glucose, in koji. Amylase as well as sucrase are perhaps the principal sugar enzymes released by the mold. Comparing the results obtained by Yong and Wood (1977a) and Goel and Wood (1978), exo-amylase (or β-amylase) level was found to increase more rapidly than did endo-amylase (or α-amlyase) level after spore germination. However, the former enzyme displayed a gradual decline in activity towards the end of the koji stage, whereas the latter showed a further increase. Both enzymes suffered from a sharp loss in activity as the moromi stage began. Following the decrease in the activity at the beginning of the fermentation was the usual pattern of fairly rapid increase at the first three or four days of incubation. From thereon, exo-amylase level remained steady for the rest of the fermentation while endo-amylase had another distinct loss of activity between the twelfth and twentieth days of incubation (Figure 6 and 7).

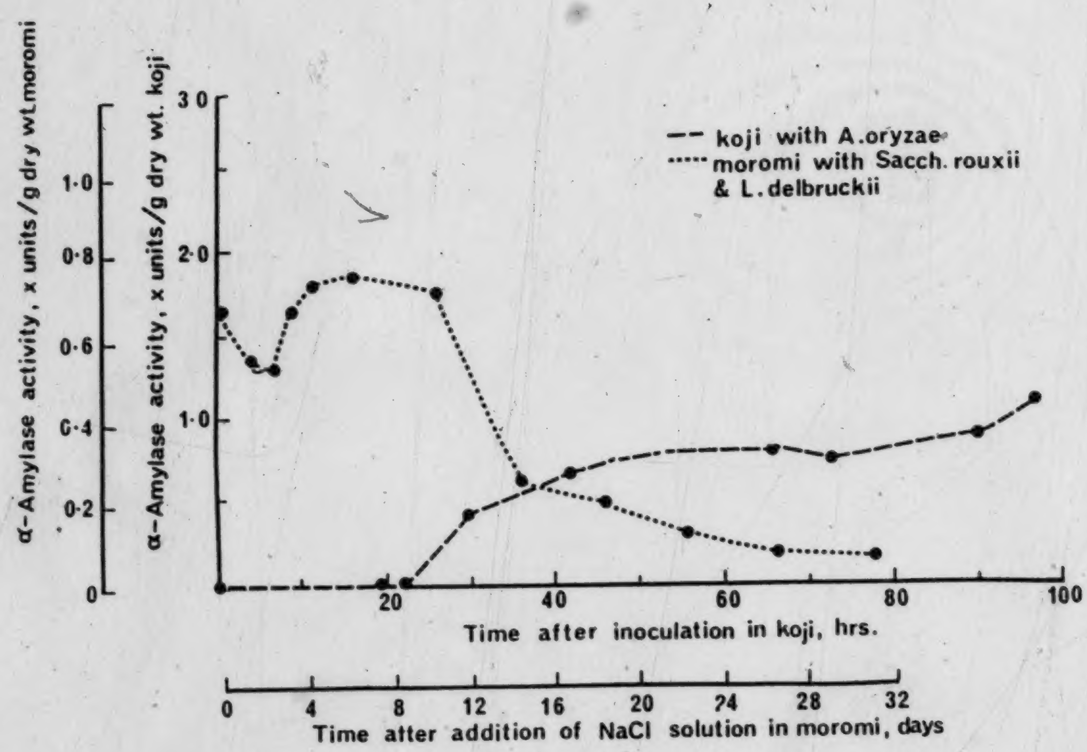


Figure 6 α- amylase in koji and moromi
After Yong and Wood, 1977a,b

The α-amylase has an activity of one x unit per gram, when 25.0 mg of that preparation reacts on 1 g dry weight of starch in a total vo,lume of 55 mL, a temperature of 30 C and a pH of 6.0, so that the achroic point is reached in 15.0 minutes.
X units per g = 15/2tc, where t = time to the achroic point ( min );
c = concentration of the extract in g per 100 mL

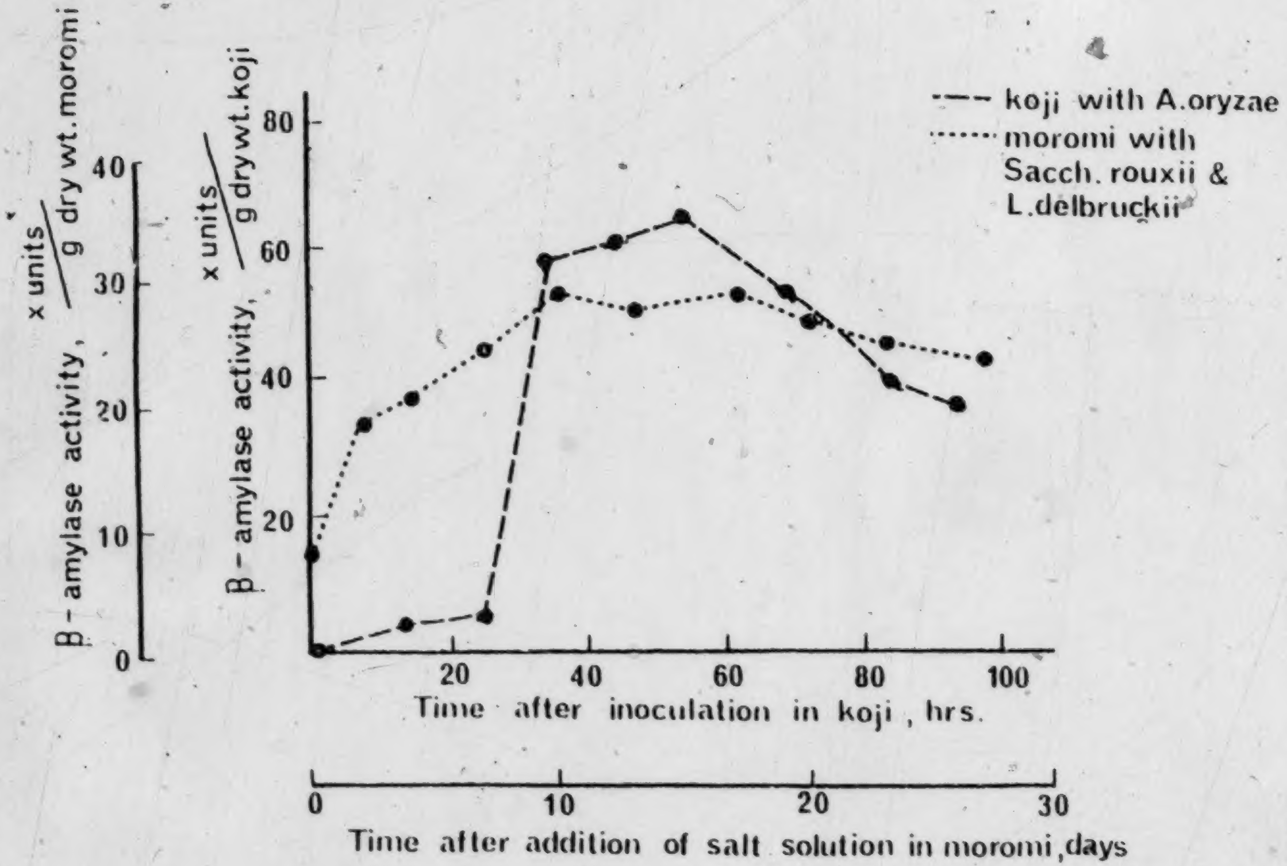


Figure 7 B-amylase in koji and moromi
After Goel and Wood, 1978

*β-amylase unit was that amount of enzyme which produced 1.0 mg of reducing sugar calculated as glucose under the experimental condition.

According to Yong and Wood (1977a), sucrase played an essential role in enzymatic hydrolysis when the endo-amylase activity was barely detected during the first twenty h of koji incubation. Results obtained by various workers suggested that besides sucrase, substantial amount of other enzymes were produced simultaneously during this period of incubation. At the eighteenth hour of incubation, sucrase was observed to reach a level of 1.1 units/g dry weight of koji (Yong and Wood, 1977a); cellulase was detected up to 1.1 units (Goel and Wood, 1978), maltase and amyloglucosidase were found to be 0.12 and 0.24 units respectively (Aidoo et al., 1981). Unfortunately, the above results could only be used as a general guideline for comparison of different enzyme levels, since different experimental methods were employed in detecting the reducing sugar content resulting from the hydrolysis.

Simley et al. (1976) reported the production of a-galactosidase by *Aspergillus awamori* on wheat bran culture and its use in a hollow fiber reactor to hydrolyze galacto-oligosaccharides of soy milk. The use of immobilized lactase produced by *A. oryzae* for processing whey was found to successfully improve nutritional value and reduce production cost in the beverage industry (Sprossler and Plainer,1983). Information on the activities of these two enzymes would be valuable because of the potential importance of their products in soy sauce flavor.

### 2.3.1.3 Others enzymes

Little attention has been paid to the presence of lipase, tyrosinase, ribonucleases, phosphatases etc. in koji. However, it is of practica significance to study the activities and levels of these enzymes since quality of the sauce can be greatly influenced by the product of their hydrolyses. Unlike proteases and carbohydratases, lipase does not contribute desirable flavor to the sauce. Instead lipase breaks down lipids in soybeans liberating free fatty acids which give rise to undesirable, rancid off-flavor. Therefore, a choice of different mold strains or replacement of whole soybeans with defatted soybean meal becomes one of the common modifications of the traditional process.

In addition to the flavor and aroma, the extent of browning reaction occurring in koji was found to correlate with tyrosinase level. Oba (1974) reported that maximum level of tyrosinase in koji was attained after 45 h of incubation. On the other hand, Yong (1971) found little evidence of tyrosinase action on color intensification in moromi and concluded that the development of the color was probably due to non-enzymatic reactions.

Nucleotides and nucleosides are often considered as one of the essential flavor components in foods. Production of phosphatases, deaminases and ribosidases by *A. oryzae* for the degradation of nucleotides was reported by Kuninaka et al. (1980). Phosphatases were described to firmly bind to the Aspergillus conidia and could be used as a neutral immobilized enzyme. On the other hand, deaminases acted on guanine, adenosine, guanosine, cytidine, adenosine monophosphate (AMP) and guanosine monophosphate (GMP) while ribosidases degraded nucleotides by acting on adenosine, inosine, guanosine and xanthosine.

### 2.3.2 Changes Taking Place in Koji and Moromi

Sauce fermentation is a complex process which involves biochemical breakdown of different substrates. As discussed above, the koji stage is dominated by the growth of Aspergillus molds, which provide hydrolytic enzyme such as extracellular protease and saccharogenic enzymes. With these enzymes, proteins and polysaccharides are broken down into small peptides, free amino acids and simple sugars, hence providing the distinctive flavor and aroma to the sauce.

The sequence of carbohydrate hydrolyses still remains vague. Chromatographic analysis of koji extract indicated that sucrose was converted within 24 h of incubation to glucose and fructose, and maltose started to disappear after 24 h of incubation (Aidoo et al. 1981). This was supported by the finding of substantial amount of sucrase at twentieth hour after mold inoculation (Yong and Wood, 1977a). Figure 8 indicates a continuous rise of reducing sugar level from the beginning of the incubation. The rise was followed by a subsequent decrease. Towards the end of the incubation, there was a second increase, then the reducing sugar content remained relatively stable thereafter.

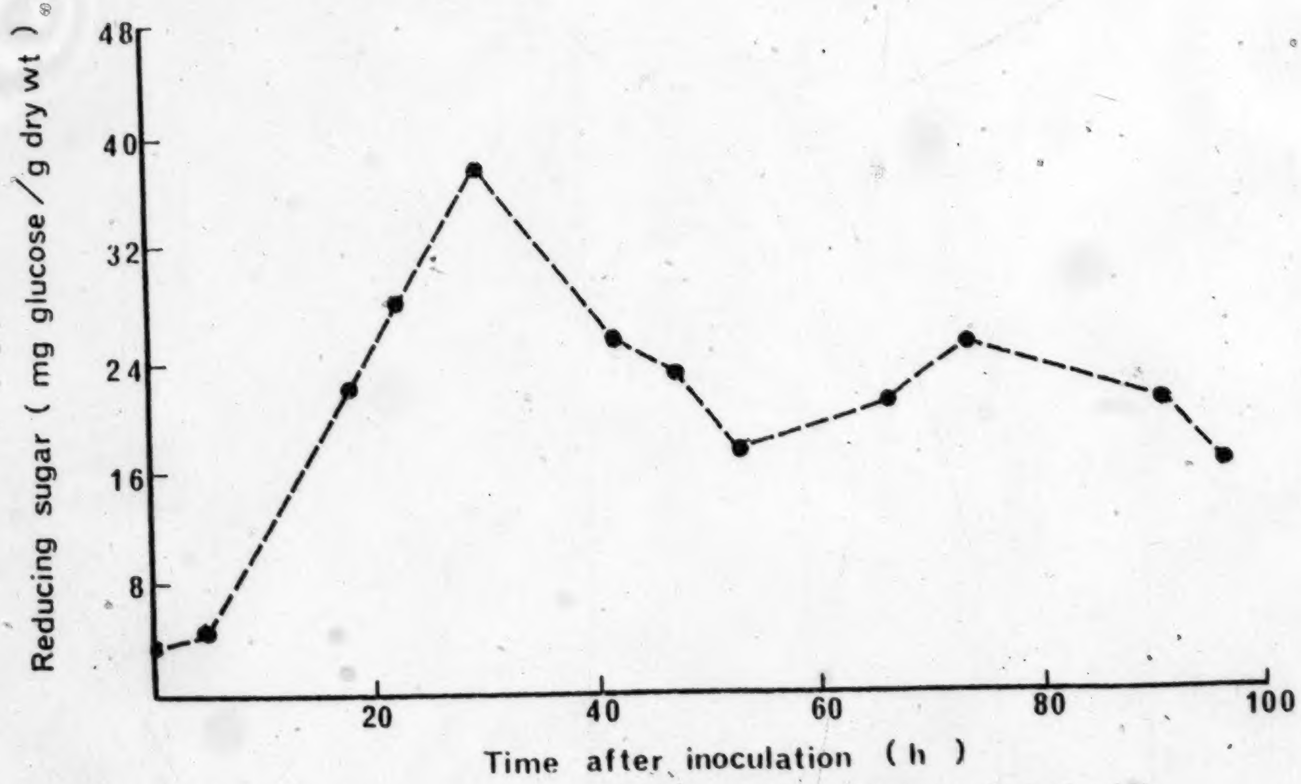


Figure 8 Reducing sugars in koji inoculated with
A. oryzae strain 1989.
S( After Yong and Wood, 1977a

Changes in total soluble nitrogen, amino nitrogen and ammonia levels are shown in Figure 9. The continual increase of total soluble nitrogen throughout the entire incubation period was consistent with the protease level observed in the koji. The amino nitrogen level fluctuated during the course of the incubation, showing no direct correlation with either the total soluble nitrgen or the protease levels.

The production of ammonia in koji is regarded as critically important to the quality of the sauce, since ammonia concentration renders the sauce unacceptable. The amount of ammonia in koji increased rapidly from about the thirtieth hour of incubation until the eightieth hour when it remained unchanged thereafter.

Since most of the important enzymes reached their optimal activities at about seventieth hour of incubation, when the reducing sugar, total soluble nitrogen and amino nitrogen levels were high, but the ammonia level was still relatively low, it was a common practice for soy sauce makers to terminate the koji after 72 h. If the koji allowed to age excessively, profuse sporulation will occur giving rise to a harsh, moldy smell to the product. The koji is terminated by the addition of salt which inhibits further mold growth.

Despite the high temperature and salinity, activities of proteases and saccharogenic enzymes remained detectable throughout the fermentation . These enzymes continued to increase reducing sugar and total soluble nitrogen contents

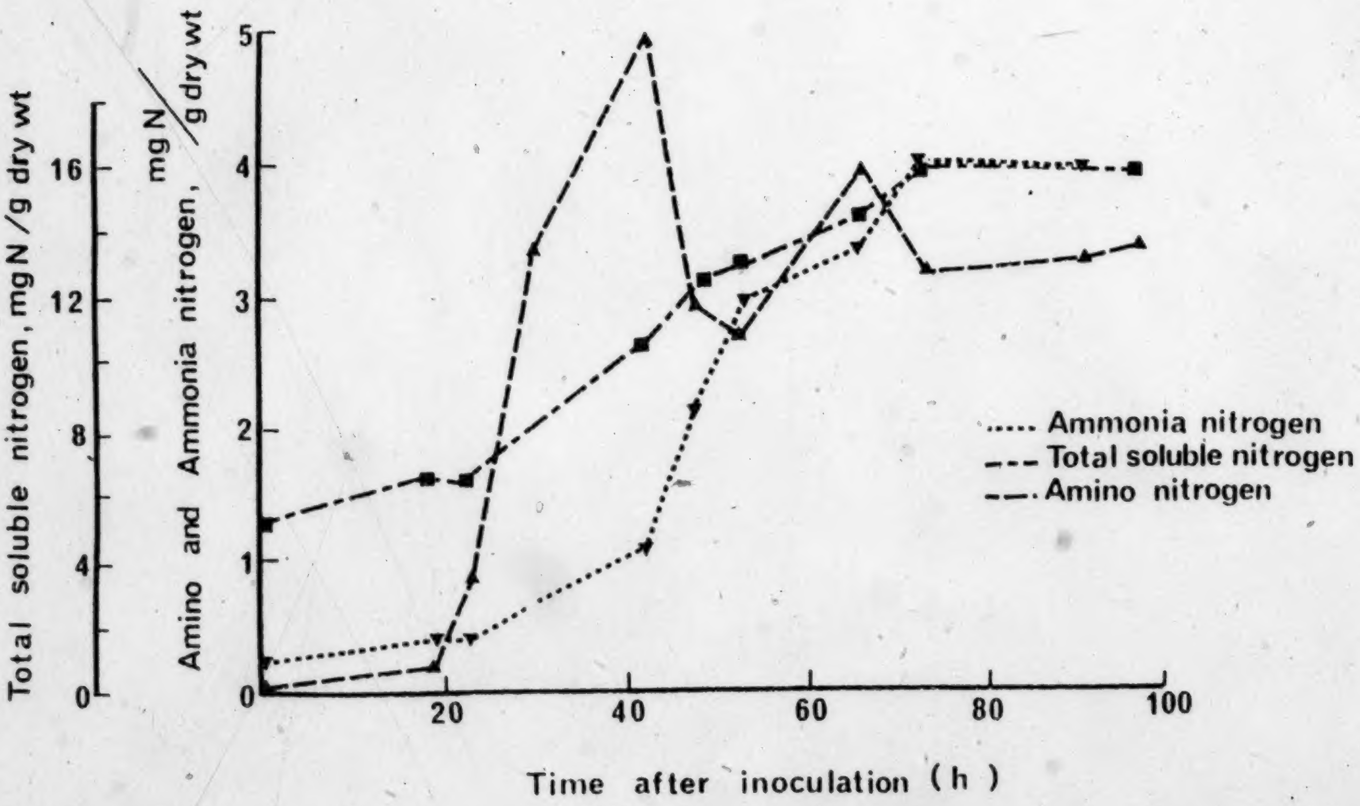


Figure 9 Total soluble nitrogen, amino nitrogen and ammonia nitrogen in koji inoculated with A. oryzae strain 1989. After Yong and Wood, 1977a

(Figure 10 and 11). Introduction of lactic acid bacteria to moromi would lead to the metabolism of simple sugars to organic acids (essentially lactic acid) and small peptides to amino acids. The production of these acids is responsible for the gradual lowering of pH. Inoculation of yeasts would result in the hydrolysis of starch to simple sugars and probably further to alcohol.

### 2.3.3 Microbiology in Moromi Mash

Fermentation of moromi is even more complex than the enzymatic reactions in koji stage. Many different micro-organism have been isolated from the mash, among them, salt-tolerant homofermentative lactic acid bacteria and yeasts are of paramount importance. The high salt concentration, around 18%, essentially restricts the growth to a few desirable osmophilic bacteria, e.g., *Pediococcus halophilus* , *P. cerevisiae*, *P. soyae* and *Lactobacillus delbruckii*. Some common contaminants such as Micrococcus and Bacillus species are sometimes found in the mash (Matsumoto,1925; Ishimaru,1933 and Sakaguchi,1958). Salt tolerant yeast such as *Saccharomyces rouxii*, *Zygosaccharomyces soyae Z. major*, *Torulopsis versatilis* and *T. etchelsii* are also found to grow successfully in the soy mash (Lodder and Kregervari Rij, 1952). Film-forming yeasts which are harmful to the keeping quality of soy sauce have also been observed. These include *Z. salsus*, *Z. japonicus* and Pichia species (Takahasi and Yukawa, 1911 and 1914).

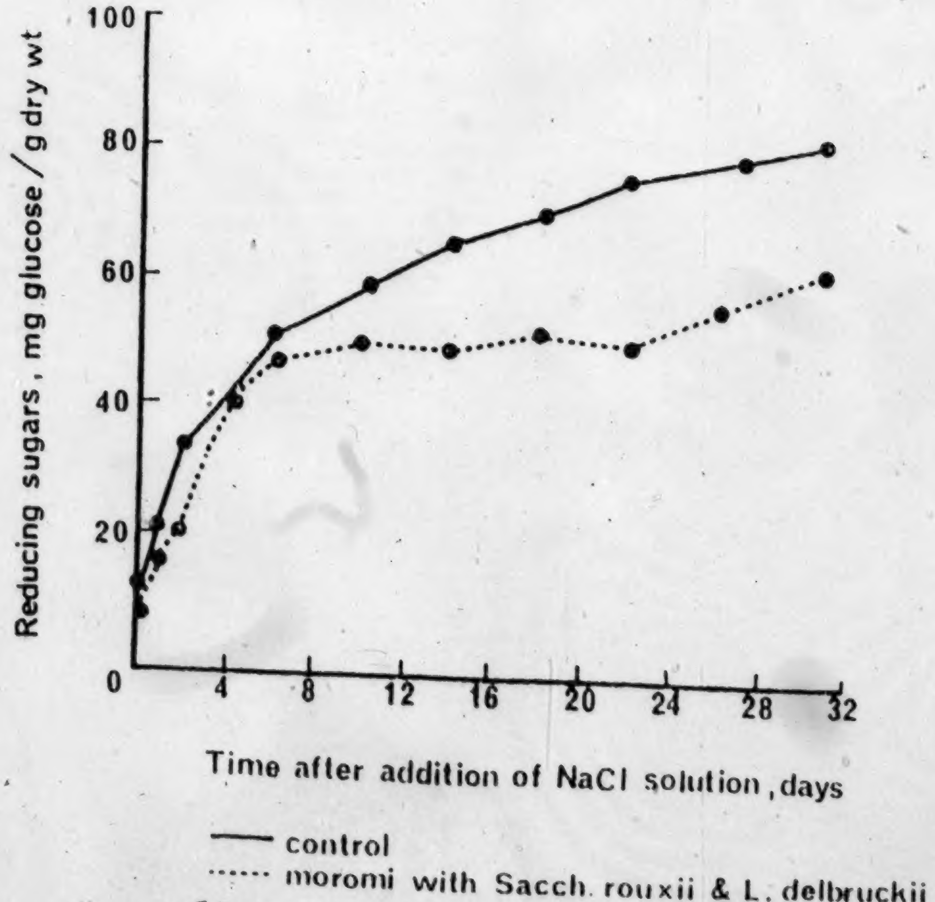


Figure 10 Reducing sugars in moromi.
After Yong and Wood, 1977b

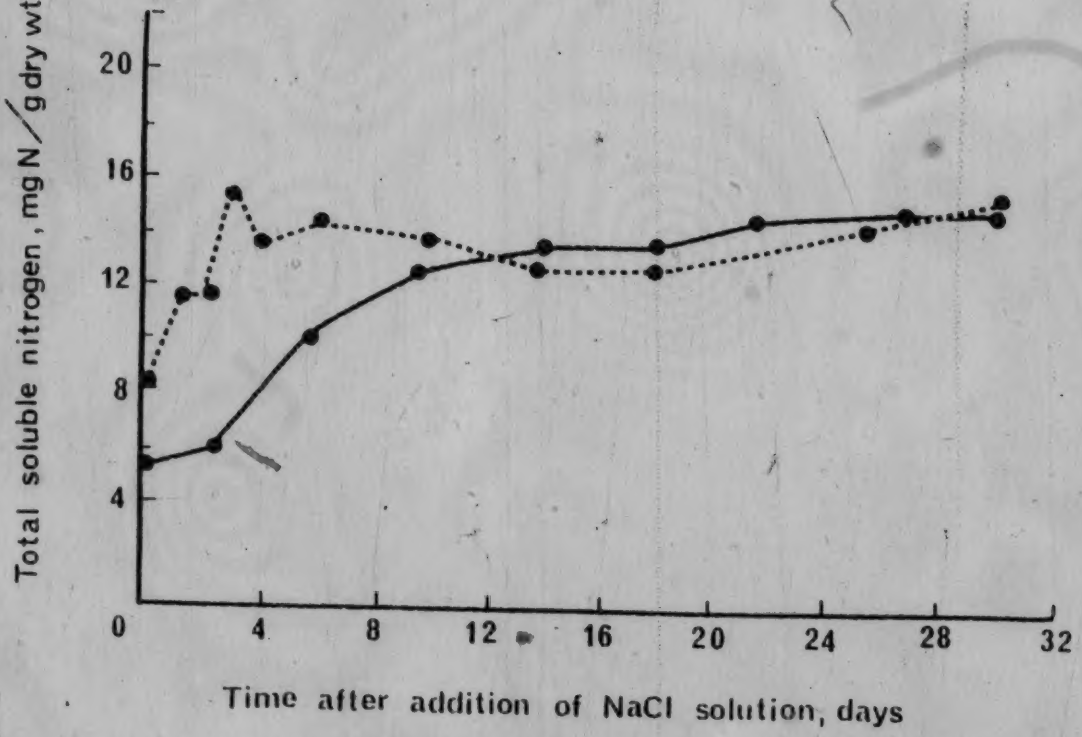


Figure 11 Total soluble nitrogen in moromi.
After Yong and Wood, 1977b

The sequence of the microbial succession in moromi has been reported by Yong and Wood (1976). They inoculated the 'trained' *L. delbruckii* and *S. rouxii* into a sterile soy mash or moromi aseptically prepared in their laboratory, and quantitated the number of microorganisms throughout the entire fermentation. These experiments showed that there was a natural gradual decrease of pH in the mash from 6.5 to 4.5 even without the presence of any detectable bacteria. Mash inoculated with lactic acid bacteria and yeast had more rapid decrease in pH. Initial viable count of *L.delbruckii* at $6.8 \times 10^4$ cells per gram dry weight of the soy mash increased to $2.5 \times 10^7$ after two days, and remained at this level for only two days before it began to decrease. The final viable count of the bacteria was found to be $1.0 \times 10^4$, after 32 days of incubation, some 700 times lower than the original inoculated level. The number of *S.rouxii* declined at the beginning from $4.6 \times 10^7$ to $1.95 \times 10^5$ followed by an increase to $7.55 \times 10^7$ on the fourteenth day of incubation when the pH was around 5.0. Thereafter, yeast dominated the brine fermentation (Figure 12).

If *S.rouxii* was inoculated alone, it initiated growth at pH 6.0 after four days, but did not rise rapidly until pH reached 5.5. It subsequently attained a relatively steady population of $1.4 \times 10^8$ after thirteen days.

On the other hand, *L. delbruckii* alone increased ninety times its original number ($3.8 \times 10^4$ cells per gram dry weight of soy mash) after four days. It then remained at

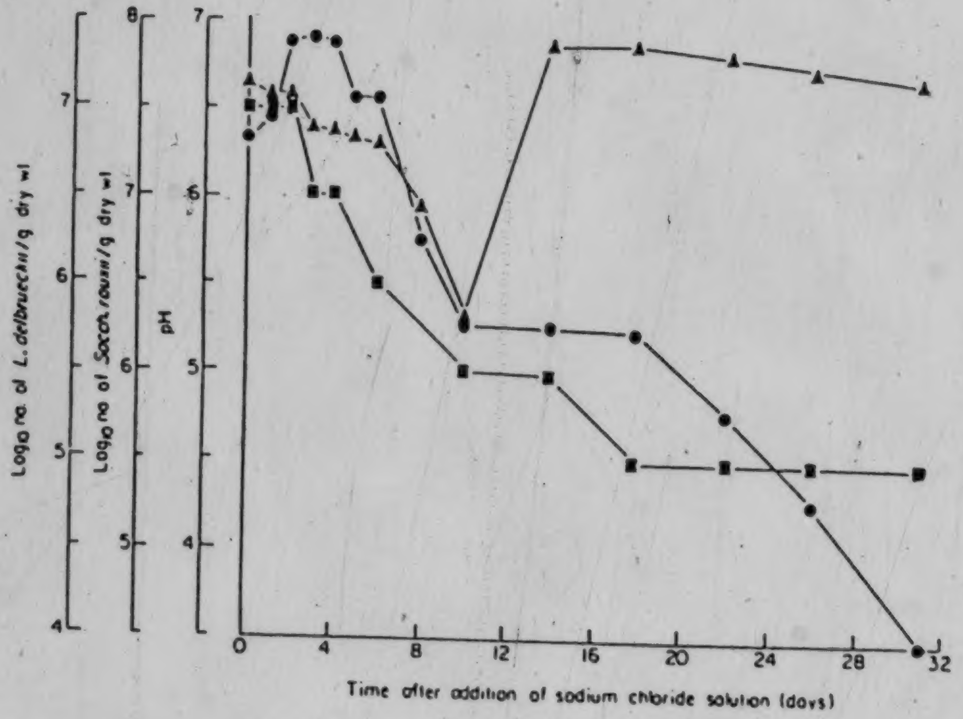


Figure 12 Viable counts of L. delbruckii ( ● ), Sacch. rouxii ( ▲ ) and pH ( ■ ) in soy mash.
After Yong and Wood, 1976

this level for four days before decreasing to the final count of only 5.6 x $10^3$ cells per gram dry weight of the sample. PH dropped most rapidly in this sample than any of the above combinations.

Another striking discovery by Yong and Wood (1976) was that when the mash was artificially soured with lactic acid to pH 4.5 and then fermented with yeast, the lag period of the yeast was shorten to one day. An optimal level at 1.4 x $10^7$ cells per gram dry weight of the mash was attained after twelve days. It was also interesting to note that the lag phase was more prolonged for yeast in medium acidified with HCl at 18 and 20% sodium chloride concentration than in a medium acidified with lactic acid at the same salt concentration (Yong et al., 1978). Sauce produced by this method had desirable quality comparable to that inoculated with both microorganisms. This implied that moromi fermentation involving only yeast could give as good a quality of product even if lactic acid bacteria were omitted. Therefore, more attention will be paid on the factors affecting soy yeast growth in this review.

Several factors such as pH, salt concentration and prolificness of lactic acid bacteria affect yeast growth during the brine fermentation. The pH range for the growth of soy yeast in sodium chloride free medium is very wide (pH 3 - 7), while in high salt concentration, profuse growth of the yeast is limited to only low pH (pH 4.0 - 5.0) (Onishi and Saito, 1961). Increasing salt concentration, which is the case in brine fermentation, exponentially extends the lag phase of the yeast. That is, a further 2% increase from 18% to 20% will prolong the lag growth phase tremendously to more than 120 h. Moreover, the total amount of yeast growth decreases with increasing salt concentration (Yong et al., 1978). All these corroborate the phenomenon observed by Yong and Wood (1976) in their study that yeast did not initiate its growth until the pH dropped below 5.0 in such a high salt medium.

Presence of lactic acid bacteria results in a depression of yeast growth. Noda et al. (1981) in their study of the interaction between bacterial and yeast growth in brine fermentation of soy sauce, found that metabolites produced by the bacteria exhibited a strong inhibitory effect on the growth of the tested soy yeasts. Acetic acid produced by the bacteria was considered to cause the microbial antagonism in soy sauce fermentation. In fact, the inhibitory effect of acetic acid on yeast growth increases significantly as the pH of the medium decrease from 6.0 to 3.5 (Noda et al., 1981). Fortunately, lactic acid, the major organic acid produced by the osmophilic yeast, does not have any drastic inhibitory effect on the alcoholic fermentation.

In short, during the first stage of the brine fermentation, lactic acid bacteria convert simple sugars, products of previous enzymatic breakdowns, to organic acids, mainly lactic acid, which lead to the decrease of pH in the medium. The viable number of the bacteria begins to decline rapidly, presumably due to the accumulation of the metabolic products in the soy mash. When the pH of the mash reaches approximately 5.0, yeast growth commences giving rise to the alcohol fermentation. *S. rouxii* ferments glucose and maltose but not galactose, saccharose and lactose. Aerobically in high salt, *S.rouxii* converts large amount (up to 50%) of glucose into glycerol and reduces furfural into furfuryl alcohol. Both glycerol and furfuryl alcohol are important flavor constituents in soy sauce (Yong and Wood, 1974). *Torulopsis versatilis* and *T. etchellsii*, somtimes isolated at the later stage of the brine fermentation, contribute to the aged mash flavor through the production of 4-ethylguaiacol, 4-ethylphenol and 2-phenylethanol (Asao et al., 1957a; 1967).

## 2.4 Chemical Composition of Soy Sauce

### 2.4.1 Nitrogen Containing Compounds

It is generally recognized in Japan that the quality and price of shoyu are determined by nitrogen yield, total soluble nitrogen and ratio of amino nitrogen to total soluble nitrogen. Nitrogen yield is defined as the nitrogenous material in the raw substrates being converted into a soluble form through enzymatic hydrolysis during koji culturing and mash aging. High nitrogen yield is an evidence of efficient enzymatic conversion, and is also one of the criteria of high quality soy sauce. Good quality soy sauce contains at least 1.5g of total soluble nitrogen per 100 mL sauce, of which, 40 to 50% is amino acids, 10 to 15% ammonia, 40 to 50% peptides and peptones and less than 1% proteins (Yokotsuka, 1960).

Amino acids exist in soy sauce mainly in free form, though some of them are in conjugated form. Lysine, histidine, aspartic acid, arginine, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine were detected by Tamura and Aiba (1982) in their purified N-material extracted from soy sauce. Among the amino acids identified, glutamic acid and aspartic acid existed in largest quantity. Udo (1931) reported that these two acids were mostly in conjugated form, and concluded that the chief ingredient responsible for the delicious taste of soy sauce was glutamic acid and its salts.

Besides amino acids, many volatile basic compounds have been isolated from soy sauce. These basic compounds were considered indispensible to the soy sauce flavor. In 1978, Nunomura et al. identified 24 pyrazines, 5 pyridines, 2 oxazoles and 4 other compounds in a commercial soy sauce. The content of the 4 major pyrazines - methyl-pyrazine, dimethyl-pyrazine, ethyl-methyl-pyrazine and trimethyl-pyrazine, increased 2.5 times during the course of pasteurization. These pyrazines had very low odor threshold, and also gave a synergistic effect in flavoring, thus played an important role in the so-called 'heated' flavor in soy sauce. Presence of other organic bases have also been reported by many different workers. The most common base constituents in soy sauce were adenine, hypoxanthine, xanthine, guanine, cytosine, uracil, chloline, betaine. These compounds were regarded to be derived from nucleic acids, produced by enzymes present in the mold ( Yokotsuka, 1960 ).

Different analytical methods have been employed to estimate percent soluble nitrogen and amino nitrogen contents in the sauce. Both macro- and micro-kjeldahl techniques were frequently used to determine total soluble nitrogen. Selection of catalysts used in digestion became subjects of many different researches. In these studies, it was reported that almost 90% of the total nitrogen was easily converted by the ordinary Kjeldahl method in which only cuprous sulfate was used as the catalyst. Some investigators suggested the use of a combination of cuprous sulfate and dipotassium phosphate or mercuric oxide as catalysts for the digestion. These improved Kjeldahl methods help to promote the breakdown of nitrogen containing phenolic fractions which are resistant to digestion during the analysis (Yokotsuka et al., 1955 and A.O.A.C. 1971. Section 2.051).

Formol titration, ninhydrin reaction etc. have long been used to determine amino nitrogen present in the product. Nevertheless, both of the methods caused erroneous results because of the ammonia present. Sakasai and

Yokotsuka (1957) used electrometric titration and Conway diffusion method to determine the formol nitrogen and ammonia nitrogen. The difference between the formol and ammonia nitrogen was regarded as amino nitrogen.

Amount of nitrogen containing compounds present in the sauce is attributed to the type of raw materials as well as different processing practices. A general trend of using defatted soybean meal instead of whole beans began fifty years ago (Wood, 1982). The reason was not only economic, but also because of a higher ultilization of nitrogen in defatted soybean meal in the fermentation. The rate of fermentation was slower when whole bean instead of defated soybean meal was employed. It was postulated that higher ratio of defatted soybean to wheat is required should a product of high nitrogen content be produced. This is because wheat is the principal carbohydrate source while soybean is responsible for the nitrogen supply in the sauce production. However, a higher carbon to nitrogen ratio (or lower soybean to wheat ratio) encourages mycelial growth and higher proteolytic activity. A maximum proteolytic activity was observed when wheat and defatted soybeans were used in equal amount (Yokotsuka, 1960).

Processing techniques also affect the changes in nitrogen constituents in the sauce. Digestibility of proteins is affected by cooking time, steam pressure and moisture content used in preparing the raw material. Noda Soy Sauce Co. Ltd. (1955) claimed that total nitrogen in soybean was best ultilized when the beans were soaked in water for 10 to 12 h at room temperature and autoclaved at 10 to 13 psi for about an hour. Immediately after cooking, the material was cooled to below 40 C. This method has since been adopted in Japan.

In 1955, Ohara and Moriguchi studied the various conditions in preparing the moromi mash in order to improve the degree of ultilization of total and amino nitrogen of the raw materials. They found that lower salt concentration in the mash would benefit the fermentation as well as protein digestibility. However, too low a salt concentration would also lead to the risk of microbial contamination. The extent of mash aging also greatly influenced the soluble nitrogen yield, especially glutamic acid content. Mash aged for 10 to 11 months resulted in highest total soluble nitrogen and amino nitrogen contents. Glutamic acid, on the other hand, reached maxium concentration after 15 months of aging after which it started to decrease (Udo, 1931).

It must be emphasized that total soluble nitrogen, amino nitrogen and amino acids contents are directly related to the sensory quality of soy sauce. Indeed, a multivariate analysis comparing different factors affecting shoyu quality indicated that nitrogen containing compounds were ranked the most important factor influencing the organoleptic property of the sauce (Mori, 1979).

### 2.4.2 Carbohydrates

Sugar content of soy sauce is usually expressed as glucose equivalent. About 3 - 5% reducing sugar was found in most of the commercial soy sauce except the shiro type, which had a distinctively high reducing sugar content of up to 20%. This is not suprising since a high percentage of wheat is used in preparing the shiro koji. Glucose, galactose, arabinose and xylose are the four major sugars isolated from soy sauce. Two sugar alcohols, deriving from mannitol and glycerol, and one non-reducing oligosaccharide were also identified (Hamada et al. 1956).

Hitherto, none of the methods which include colorimetry using chromogenic reagent, and titration using Fehling reagents and sodium thiosulfate, has been found satisfactory in estimating sugar level in the sauce. (Somogyi, 1951 and and A.O.A.C. 1975, Section 31.038 - 40). Interfering substances in soy sauce such as proteins, amino acids, and other reducing compounds cause errors in these analyses. Attempts have been made to remove proteins before the analysis. However, this procedure alone did not seem to be effective in overcoming the problem since many substances are involved (Kandachi and Nishi, 1949)

Although sugar itself does not seem to play a dominant role in taste and aroma of the sauce, its derivative compounds ameliorate the flavor profile. Non-enzymatic browning or Maillard reaction is a major contributor of pleasant organoleptic characteristic as well as the traditional dark brown color of the sauce. Soy sauce with too light a color is generally considered to lack 'body' by the orientals. Five Amadori compounds: fructose-alanine, fructose-valine, fructose-glycine, fructose-leucine and fructose-isoleucine were isolated and identified from soy sauce. All these compounds exhibited remarkable browning in the presence of oxygen and $Fe^{2+}$ or both $Mn^{2+}$ and $Fe^{2+}$. $Mn^{2+}$ itself had no effect on the oxidative browning of the Amadori but together with $Fe^{2+}$, synergistically accelerated the reaction. Rearrangement of the Amadori compounds was also enhanced in the presence of other amino acids. Furthermore, the degree of browning caused by $\alpha$-hydroxycarbonyl group was insignificant as compared to that caused by the oxidative browning of Amadori compounds. It was, therefore, concluded that the oxidative browning of soy sauce was accelerated appreciably by the presence of $Fe^{2+}$ and $Mn^{2+}$ whose concentrations in the sauce were about 40 and 30 p.p.m. respectively (Hashiba, 1976,1978).

Another significant role of sugar in soy sauce is to supply nutrient to the yeast for the production of alcohols and their derivatives. These fermentation products play an essential role in aroma which will be discussed in Section 2.4.5..

### 2.4.3 Acids and Related Compounds

Experiment on quality evaluation and chemical composition of soy sauce conducted by Onaga et al. (1957)

revealed that sauces with best rated flavor and aroma had high total acidity. These acids might be derived from the metabolic products formed during fermentation or from some of the amino acids such as glutamic acid and aspartic acid as described earlier. The total titratable acidity measured from seven commercial samples varied from 6.13 to 19.97 milliequivalent NaOH per 100mL sauce.

Acid shown to be related to the desirable aroma, taste, storage and color quality of the sauce include lactic, acetic, succinic and phosphoric acid. Formic and levulinic acid, their presence generally regarded as undesirable, are the major organic acids present in chemical or semi-chemical soy sauce. Minor organic acids such as propionic, butanoic, malic, citric, glycolic etc. have also been isolated from the product. The organic acid content of soy sauce determined by Ueda et al. (1958) is listed in Table 2 . It was also found that arginine, histidine, lysine, putrescine, cadaverine and choline, when conjugated with succinic acid and glutamic acid, gave delicious and sweet taste, while salts of formic, acetic, lactic and phosphoric acid generally tasted bitter.

The acids predominant in moromi in the early brewing stage are citric acid and malic acid. As fermentation proceeds, lactic acid bacteria such as *Pediococcus halophilus*, convert glucose to lactate while metabolizing citric and malic acids to produce primarily acetic acid. The ability to metabolize these acids differs from strain to

Table 2. Organic acid contents of soy sauce[a]

| Sample no. | 1 | 2 | 3 |
|---|---|---|---|
| Butyric acid | 1.03 | 2.39 | 4.30 |
| Isobutyric acid | 0.66 | 4.56 | 1.20 |
| Unknown | - | 0.80 | |
| Propionic acid | 11.89 | 18.60 | 2.64 |
| Levulinic acid | 172.28 | 153.93 | 1244.51 |
| Acetic acid | 157.92 | 114.41 | 122.54 |
| Pyruvic acid | 8.81 | 4.20 | |
| Formic acid | 26.13 | 18.14 | 287.80 |
| α-Ketoglutaric acid | 1.68 | 4.26 | 4.67 |
| Succinic acid | 77.15 | 33.32 | |
| Lactic acid | 1175.25 | 1221.73 | 29.53 |
| Pyroglutaric acid | 8.81 | 4.20 | |
| Glycolic acid | 18.32 | 8.10 | 4.16 |
| Malic acid | 4.21 | 3.05 | 3.28 |
| Citric acid | 7.49 | 13.04 | 25.27 |

a After Ueda et al. (1958)
Data expressed as mg/100ml
b No.1 and No.2 are fermented soy sauce, and no.3 is hydrochloric acid hydrolyzate of soybean meal

strain. From the first to the ninth month of fermentation, the overall organic acid content in moromi increases. Lactic acid reaches its optimal level in 3 to 4 months while acetic, propionic and formic acids attain their maximum content by the eighth month. Towards the end of the fermentation, there is a decrease in most of the volatile organic acids (Kanabe and Uchida, 1982).

The commencement of yeast fermentation at the late mash aging period marks the assimilation of ethanol and carbon dioxide at the expense of glucose. In 1980, Yong et al. suggested four glucose fermentation patterns under various yeast culturing and mash aging conditions. They reported that only yeast having been cultured at acidic pH (4.5) and inoculated in acidic mash (4.5) could successfully produce ethanol and carbon dioxide. Cells grown in acidic media and introduced into neutral mash or vice versa were apt to produce volatile organic acids such as acetic and formic acids. More acids would be produced if yeast grown in neutral medium was inoculated into neutral moromi mash. Ethyl acetate which was one of the natural preservatives and flavors of the sauce was synthesized aerobically by yeast at the later stage of soy brewing. A large amount of ethyl acetate was produced when ethanol was metabolized in conjunction with glucose (Yong et al., 1981)

The most important part of fragrance characteristic of soy sauce seems to exist in acidic state of pH 4.6 to 5.0. Nunomura et al. (1980) isolated 2 volatile acidic fractions and 7 non-volatile concentrates directly from $CH_2Cl_2$ extract. The above fractions were subjected to gas chromatography and combined gas chromatography-mass spectometry analyses. Ninety three components were identified, of which, there were 15 alcohols, 15 carbonyls, 4 lactones, 13 acids, 9 esters, 6 furans, 3 furanones, 5 pyrones, 7 phenols, 2 sulfur-containing compounds and 14 other compounds. The acids isolated were acetic acid, propionic acid, butanoic acid pentanoic acid, methyl pentanoic acid, methyl butanoic acid, hexanoic acid, benzoic acid, etc.. Most of these acids were esterified with ethyl or propyl groups. Some volatile acids which were not identified by Nunomura et al. (1980) were identified by other workers. These acids include oxalic acid, vanillic acid, ferulic acid, syringic acid and trimethyl-gallic acid. The origin of these acids was believed to be from the lignin substances in the raw materials (Asao et al., 1957a,b). Other acidic flavorous compounds such as phenols, furanones, pyrones, etc., will be discussed in details in Section 2.4.5.

### 2.4.4 Principle Flavor Components

Studies of volatile and non-volatile flavor components in soy sauce are of interest both to manufacturers and researchers. The earliest research on flavor chemistry in soy sauce was conducted by the Japanese in 1887. With the efforts of many investigators throughout the succeeding century, over 300 kinds of volatile flavor constituents have been isolated from the sauce. These include 51 carbonyls, 24 organic acids, 36 nitrogenous compounds, 41 esters, 31 alcohols, 3 acids, 11 sulfur containing compounds, 17 phenols and 62 others (Yokotsuka, 1981). Most of the desirable volatile flavor components exist in acidic pHs between 4.6 to 5.0. It was found that the fragrance of soy sauce disappeared once the sauce was neutralized with alkali.

In 1980, Nunomura et al. isolated and identified 93 compounds from the acidic fractions of soy sauce as stated in the previous section. 2,3-butanediol was found to be the major component in the sauce bouquet. 4-hydroxy-5-methyl-3(2H)-furanone (HMF), 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone (HEMH) and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) which possessed strong caramel-like odor and soy sauce-like aroma were isolated. These furanones were probably formed through heating of sugars in the raw materials during the manufacturing process. The very low threshold values of HEMF and HDMF (0.04 p.p.b. in water for both) together with their high concentration in the sauce of about 100 - 200 p.p.m. and 10 p.p.m., respectively, made them very important components in soy sauce flavor (Ohloff, 1978; Nunomura et al., 1976b). HMF, on the other hand, which existed in small amount in raw sauce, increased to about 200 p.p.m. upon pasteurization.

Pyrones such as isomaltol and maltol, well known flavor enhancers, were also isolated from the sauce (Nunomura et al., 1976a).

According to Yokotsuka et al. (1967a,b), 4-ethyl-guaiacol (4EG) ameliorated the salty taste of soy sauce and also marked the distinctive brand character of a shoyu. The most popular brands of Koikuchi-shoyu in Japan contained about 1.0, 1.8 and 2.1 p.p.m. of 4EG which were substantially higher than those of the less-accepted brands. Other phenolic compounds such as vanillin and vanillic acid, reacted with organic acids to form phenolesters and contributed as an essence to the flavor profile.

The reminiscence of the head space aroma from freshly fermented soy sauce was mainly attributed to ethanol, isoamyl-alcohol, isovaleraldehyde, isobutyraldehyde, diacetals of these aldehydes, ethyl-acetate as well as a minute amount of dimethyl-sulfide (Sasaki and Nunomura,1981; Yoshida et al., 1980).

It is evident, therefore, that the flavor chemistry of soy sauce is an extremely complex subject. Different chemical compounds are produced , reacted and conjugated with one another during the manufacturing process. Food researchers have been endeavoring to isolate and identify the flavor constituents in the sauce and trying to correlate them with sensory characterstics of the sauce. Aishma (1977 a,b and 1981) studied the relationship between soy sauce gas chromatographic patterns and the ranks of sensory quality of the sauce samples by means of multiple regression and stepwise multiple regression analyses. He reported that trans-2-hexene-1-ol contributed to pleasant aroma while isobutyric acid to the undesirable smell in the sauce. Multiple regression models have also been used to estimate the contribution of each individual GC-isolated flavor component to the organoleptic and aromatic characteristics of the sauce.

### 2.4.5 Color

Soy sauce, like many other brewed product is characterized by its distinctive dark red color. The browning of soy sauce is suspected to be both enzymatic and non-enzymatic. Non-enzymatic browning is largely due to the Amadori compounds formed by the reaction between sugars and amino-compounds (Hashiba et al., 1978). However, the reaction mechanism of the browning process has not yet been thoroughly understood. Hitherto, only some observatory facts about soy sauce browning are obtained; the actual mechanism of the browing process still remains unknown. One of the findings indicated that the browning rate of soy sauce depends on the extent of fermentation during mashing period and on the content of non-amino acid nitrogenous materials. Presence of reducing agents, in the case of mash aging of moromi, depressed the non-oxidative browning reaction (Okuhara and Saito, 1970 a,b). Similar finding on the decolorization of raw soy sauce by reduction under a simulated fermentation condition suggested that soy sauce which had been kept under anaerobic conditions by favorable fermentation during mash aging was light in color and slow in browning (Okuhara and Saito, 1970c). Futhermore, an increase in the volume of mashing saline decreased the browning rate (Okuhara et al. 1971).

On the other hand, Okuhara et al. (1971) reported that the browning rate of soy sauce was promoted by the addition of peptide solution which not only brown themselves by reacting with sugars in the mash, but also greatly accelerated the browning reaction between amino acids and sugars.

In 1981, Hashiba et al. found that Amadori compounds were 10-100 times more reactive in browning than parent materials, amino compounds and sugars, especially in the presence of oxygen and iron. Moreover, oxidative browning of Amadori compounds derived from peptides was more eminent than those from amino acids.

Constituents composed of the Amadori compounds were also found to determine the color formation and its intensity. For example, red color of the sauce was considered to be affected by the sugar moiety of the Amadori compounds, whereas, the color intensity decreased with increased side chain of the amino acids constituting the Amadori compounds.

Color is associated with the visible waves which lie between 400 to 700 nanometers, from violet to red ends of the spectrum, respectively. In order to standardize the color measurement for scientific research and accuracy, different systems have been proposed. These include the 1931 CIE Standard Observer and Hunter L,a,b color co-ordinate systems. The former system is based on the three color response mechanisms of the human eyes. Specific numerical values (X, Y, Z) are assigned to these three color-matching responses. However, the CIE scales do not provide reasonably uniform estimates of perceived color intervals or color relationships. The latter system gives uniform color scales and is based on the opponent - color theory. This theory presumes that color perception by human eye corresponds to the signal-switching stage between the light receptor on the retina and the optic nerve response to the brain. Color signals are interpreted on contrasting dimensions; red (+a) is compared to green (-a) while yellow (+b) is compared to blue (-b). The third dimension is lightness (L); 100 for white and 0 for black. The units (X, Y, Z) in CIE system and L,a,b in Hunter color co-ordinate system are interchangeable by some equations. Hunter L,a,b Color Co-ordinate system is employed in measuring the sauce colors in this research because of its simplicity, convenience and world-wide acceptance.

## 2.5 Alternative Materials and Processes for the Sauce Production

### 2.5.1 Use of Other Substrates

With the ever increasing costs of labor, time and space, it is imperative that the ancient ways of soy sauce fermentation must be modified or replaced with more efficient and quality-guaranteed processes. However, most of the methods suggested are just ad hoc modifications of the traditional process. They are still time consuming, expensive and inefficient. Several attempts have been made to use different ingredients to produce sauce with quality comparable to that of the commercial soy sauce. In 1923, Church used peanut meal, instead of soybean, and wheat mixture to produce the sauce. In 1948, Tsukahara, perhaps impressed by the dark color of soy sauce, tried to produce the sauce from garbage, while Oda et al. (1949) made the sauce from acorns and wheat. Matsumoto (1964) ultilized soy hull instead of soybean. In 1966, Beans-Arcega replaced wheat with copra meal in the sauce fermentation. One of the more recent trials was done by Luksas (1971 a,b), who suggested the production of soy sauce by fermentation of whey with *Saccharomyces lactis* and *S. cerevisiae*. However, none of the above trials have found commercial success. This may be due to the unsuitability of the soybean substitutes, or the contamination of undesirable microorganisms during the fermentation.

In addition to the foregoing industrial and economic considerations, an improvement in the nutritive quality of soy sauce has also been suggested. Camirand et al. (1983), for example, have tried to exchange sodium in the sauce with potassium through either a resin-bed ion exchanger or a membrane dialyzer.

As for alternative process, the use of acid to prehydrolyze soybean and wheat in order to shorten the fermentation time has already been described in Section 2.2.3. - 2.2.4.. Another possible improvement is supplementing the koji enzymes with readily available, powerful and comparatively inexpensive pure enzymes. A challenging task of food scientists is, therefore, to develop an inexpensive process which requires very short fermentation time to produce high quality sauce from relatively cheap and abundant raw materials, preferably available locally.

### 2.5.2 Preliminary Study Using Canola Meal as a Raw Material

In 1980, Ooraikul et al. ultilized canola meal as a raw material for sauce production. They compared the conventional method adapted from Ueda et al. (1958) with the semi-chemical approach from Hesseltine and Wang (1972). Both of the methods used relied on natural fermentation; i.e., no inoculation of lactic acid bacteria nor yeast was involved. In the conventional method *A. oryzae* and *A.sojae* were used to prepare the koji. Whereas, in the semi-chemical approach, one kilogram of defatted rapeseed meal was mixed with three litres of 6% hydrochloric acid and steamed under atmospheric pressure for ten h. Its pH was then adjusted to 5.5 with sodium carbonate. Both of the processes are illustrated in the flow charts (Figures 13 and 14).

### 2.5.2.1 Results of the study

PH and total soluble solids contents of the fermenting mashes were monitored throughout the entire fermentation. In the conventional method, two rises of total soluble solids were found in the moromi stage (Figure 15).The rise during the first 10 weeks was considered to be due mainly to acid fermentation by lactic acid bacteria, with the gradual lowering of pH, and the second increase to yeast fermentation. A lag period was found between the tenth and fourtieth week of fermentation. The sustained lag period was thought to be due to slow, or even declining, rate of growth and activities of the acid producing bacteia. The inhibition of growth could be brought about by the accumulation of their own metabolites and high acidity in the moromi mash. The fermentation pattern for canola meal appeared to parallel that of soy sauce described by Yokotsuka (1960) and Yong and Wood (1976). In all, these results implied the suitability of rapeseed meal as soybean substituent.

In the semi-chemical approach, the initial total soluble solids content was higher as compared with that in the conventional method. The lag period was thus greatly shortened. Soluble solids content quickly rose from 29% to

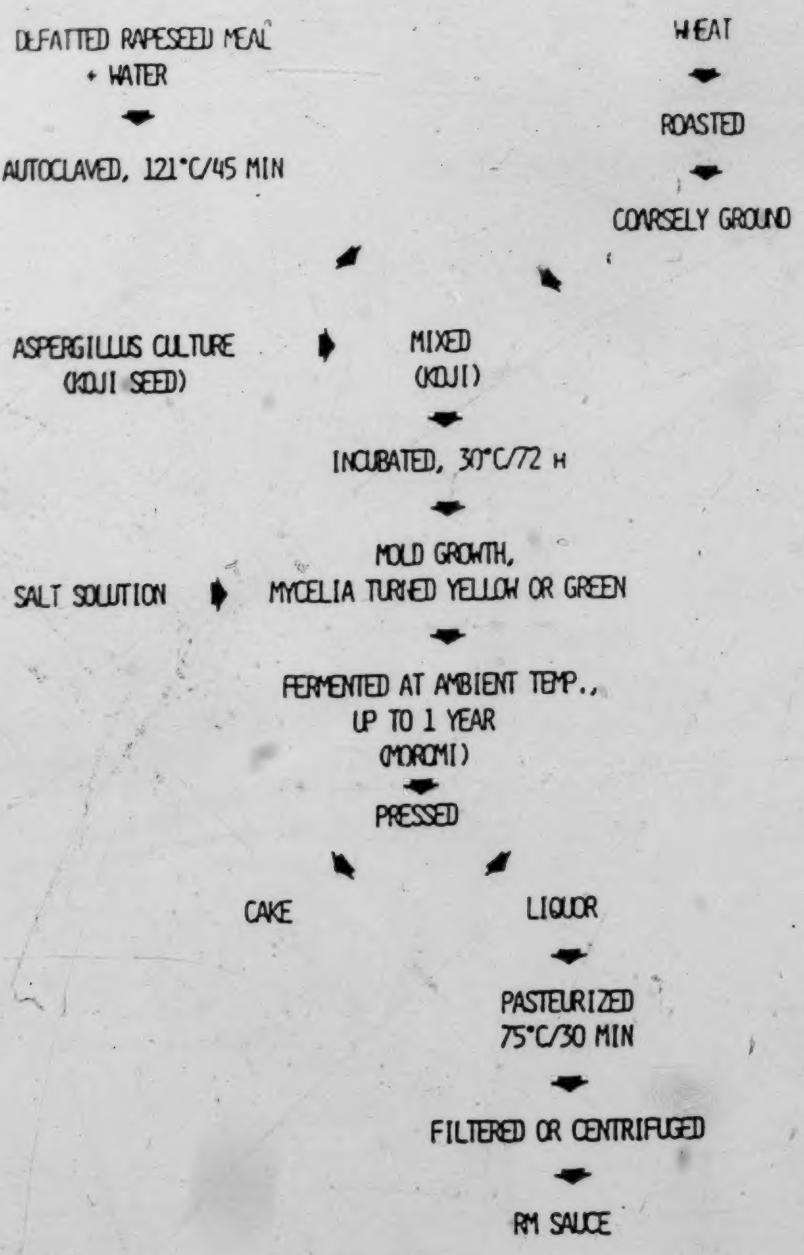

DEFATTED RAPESEED MEAL
+ WATER
AUTOCLAVED, 121°C/45 MIN
WHEAT
ROASTED
COARSELY GROUND
ASPERGILLUS CULTURE
(KOJI SEED)
MIXED
(KOJI)
INCUBATED, 30°C/72 H
MOLD GROWTH,
MYCELIA TURNED YELLOW OR GREEN
SALT SOLUTION
FERMENTED AT AMBIENT TEMP.,
UP TO 1 YEAR
(MOROMI)
PRESSED
CAKE
LIQUOR
PASTEURIZED
75°C/30 MIN
FILTERED OR CENTRIFUGED
RM SAUCE

Figure 13 Flow chart of conventional fermentation process on canoal sauce.
After Ooraikul et al. 1980.

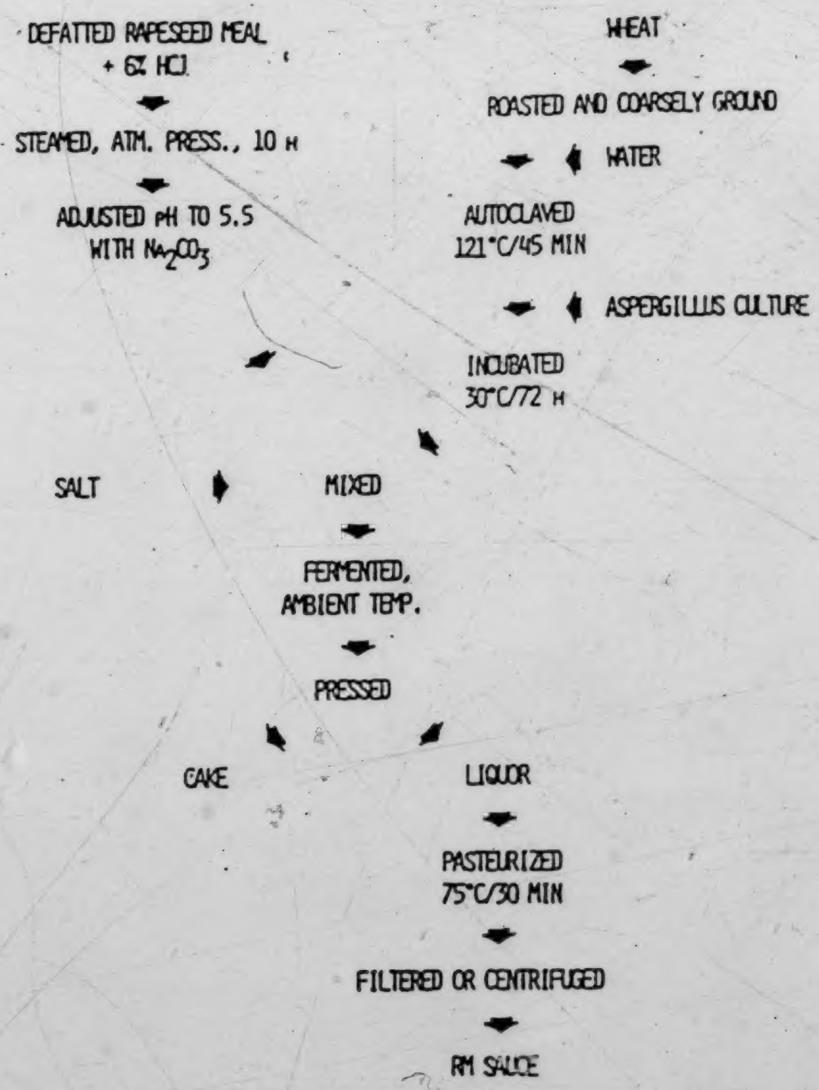

DEFATTED RAPESEED MEAL + 6% HCl
STEAMED, ATM. PRESS., 10 H
ADJUSTED pH TO 5.5 WITH $Na_2CO_3$
WHEAT
ROASTED AND COARSELY GROUND
WATER
AUTOCLAVED 121°C/45 MIN
ASPERGILLUS CULTURE
INCUBATED 30°C/72 H
SALT
MIXED
FERMENTED, AMBIENT TEMP.
PRESSED
CAKE
LIQUOR
PASTEURIZED 75°C/30 MIN
FILTERED OR CENTRIFUGED
RM SAUCE

Figure 14 Flow chart of semi - chemical process on canola sauce. After Ooraikul et al. 1980.

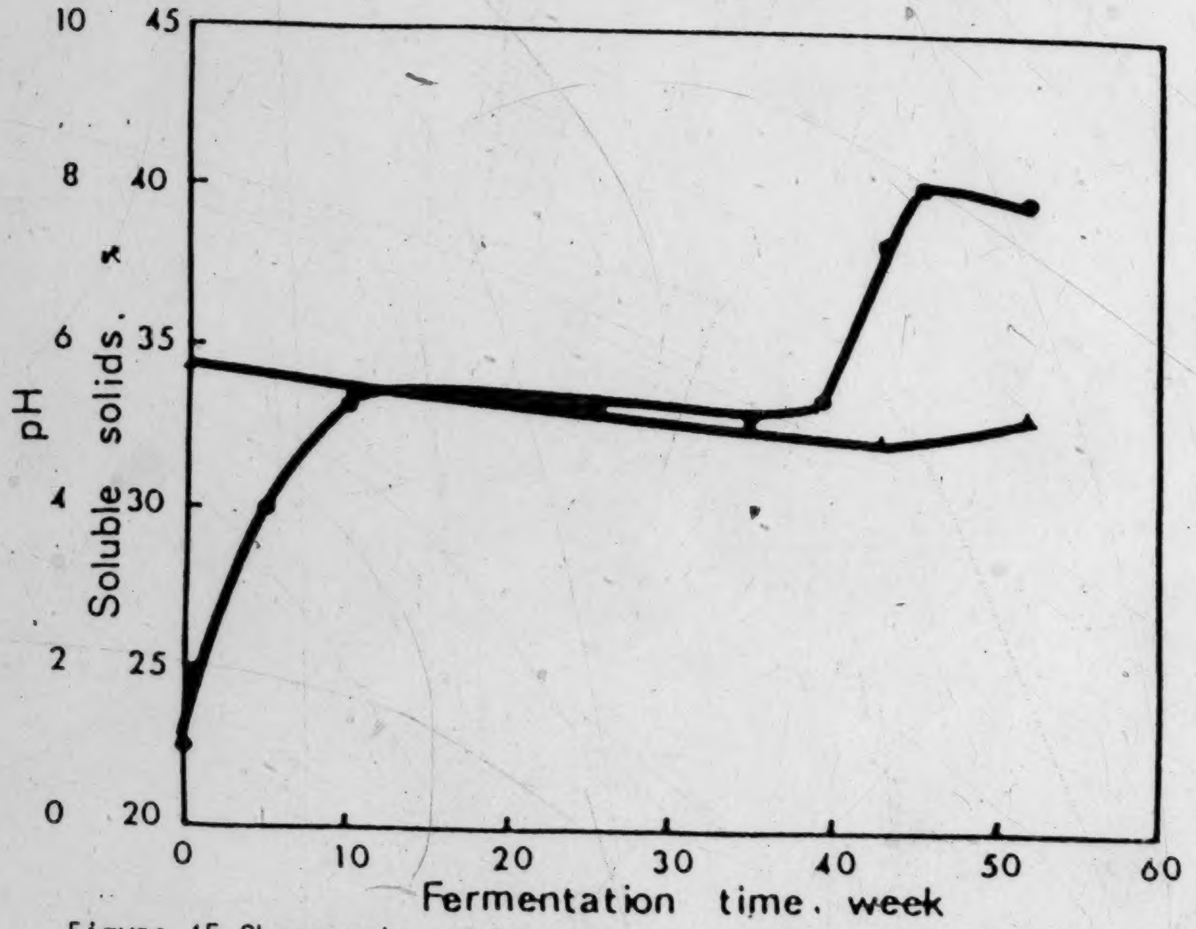


Figure 15 Changes in soluble solids and pHs during the conventiona fermentation ● soluble solids; ▲ pH
After Ooraikul et al. 1980

42% within the first month, whereas in the conventional method, it took about a year to do so (Figure 16). However, the second rise in soluble solids did not occur in the semi-chemical approach, implying that secondary fermentation by yeast did not take place.

Despite the significant increase in soluble solids, which consisted largely of amino and organic acids during fermentation in both processes, there was no corresponding sharp decline in pH. This was ascribed to the high buffering capacity of the moromi.

Sensory evaluation comparing the canola sauce and commercial soy sauce showed that both the canola sauce fermented for one year and the semichemical sauce fermented for one month were rated comparable to Kikkoman shoyu.

The results from this preliminary study indicated that the traditional fermentation used with soybean and wheat appeared to work equally well with rapeseed meal and wheat. Furthermore, the semi-chemical method was shown capable of shortening the fermentation lag period to hasten the process from one year to one month. Flavor and taste of the products were found to be comparable to those of commercial soy sauce. Conceivably, with further improvements, the use of the semi-chemical approach on canola meal in large scale industry appeared promising.

### 2.5.2.2 Problems encountered

Although the use of acid hydrolysis to pretreat canola meal prior to fermentation appears encouraging, there are

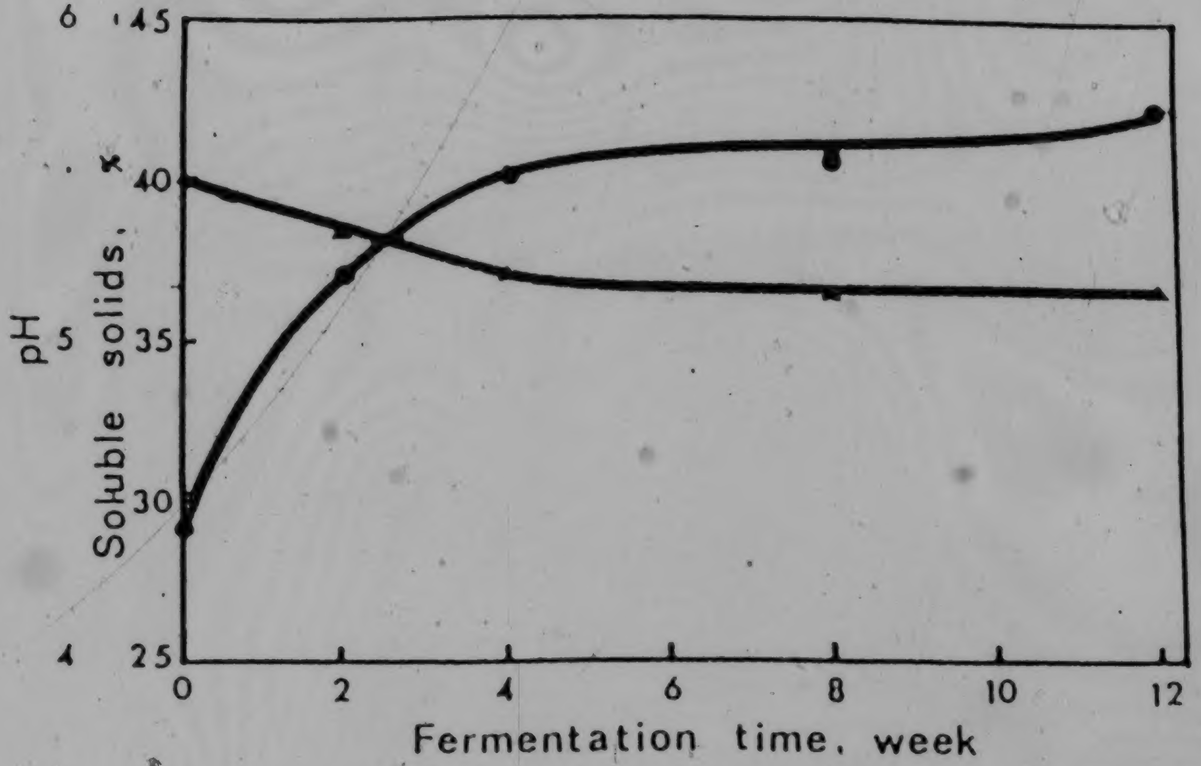


Figure 16 Changes in soluble solids and pHs during the semichemical process.
Source : Ooraikul et al. 1980.

several inherent problems with the method which can hamper the use of such treatment on industrial scale. First of all, high acid concentration is corrosive, thus necessitates the use of acid resistant processing equipment. This, of course, adds to the production cost. Furthermore, secondary fermentation involving yeast does not occur in the semichemical process resulting in the deprivation of some aromas and flavors in the product. Thirdly, food grade acid and alkali necessary for the process are quite expensive, and application of the acid may lead to the production of some undesirable substances as described in Section 2.2.5. Therefore, furhter improvement of the method to solve the said problems, or replacement of the acid with other more moderate hydrolyzing agents are necessary. The latter was chosen for the research described in this thesis.

# 3. EXPERIMENTAL

## 3.1 Optimization of total soluble nitrogen in Canola Meal-enzyme Hydrolyzate

### 3.1.1 Experimental Design

To optimize the percentage total soluble nitrogen (TSN) obtained with enzymatic hydrolysis, preliminary studies of proteolytic enzymes using the classical one factor-at-a-time approach was done. At this stage, five factors, viz., temperature, pH, enzyme-substrate ratio (E/S), solvent-meal ratio (S/M) and hydrolysis time were investigated one at a time while keeping the rest of the parameters constant. Peak values of each of the individual factors were obtained in these experiments.

Due to experimental and processing limitations, the S/M ratio and hydrolysis time were fixed at their optimal values according to the results obtained in the preliminary studies. The remaining three factors were then used in response surface optimization.

Originally, two enzyme, i.e., Alcalase 0.6 L and Neutrase 0.5 L (Novo Industri A/S, Dagavaerd, Denmark) were used in the studies. However, it became apparent after the preliminary studies that Alcalase 0.6 L was a much more efficient proteolytic enzyme for the canola meal system than Neutrase 0.5 L. Therefore, Alcalase 0.6 L was the only enzyme employed in subsequent studies.

### 3.1.1.1 One-factor-at-a-time approach

Five grams sample of canola meal was used in each of the experiments except in the study of meal-solvent ratio where 10g meal with specific amount of deionized water was used in each sample. Phosphate or carbonate buffer solutions with pre-adjusted pH were used as the solvents in the studies of temperature curve, pH curve, duration of the hydrolysis curve, and enzyme-substrate ratio curve.

Alcalase 0.6 L employed in this experiment is an endoprotease of serine type. It is a water soluble clear brownish liquid. The density of the undiluted preparation used in this experiment is approximately 1.25 $g/cm^3$. It contains 0.6 Anson units per g (AU/g) which, according to the supplier, is defined as the amount of enzyme digesting the hemoglobin under the standard condition at an initial rate liberating per min an amount of TCA soluble product which gives the same color with phenol reagent as one milliequivalent of tyrosine. The standard conditions for carrying out the hydrolysis were: 25°C, pH 7.5 and 10 min reaction time.

Technical data supplied by the company gave optimal temperature and pH for this enzyme to hydrolyse denatured hemoglobin of 60 - 70°C and 8 - 9 respectively. Complete proteolytic breakdown of wheat gluten and soy isolate was claimed to be achieved in 2 - 3 h. Therefore, in initial tests to determine optium conditions for canola system, temperature, pH and reaction time were fixed at 66°C, pH 9 and 2 h, respectively. The enzyme in dilute buffer system was found to be stable at pH 5 - 10 with temperature up to 65 °C. The stability could be enhanced if proteins or peptides were added to the system (Novo Industrias, Bagsvaerd, Denmark).

### Meal - solvent ratio

To determine optimum quantity of water required to extract soluble nitrogenous compounds from canola meal, different amounts of deionized water were used to hydrolyze 10 g of meal at 60 °C for 3 h. The ratio ranged from 1 : 3 to 1 : 25 (w/v). The mixtures were then pressed and hydrolyzates filtered and analysed for total soluble nitrogen.

### Temperature

Canola meal - enzyme mixture was incubated at temperature ranging from 50 - 80 °C with pH adjusted to 8.8 using carbonate - bicarbonate buffer. A 1 : 10 (w/v) meal - solvent ratio was used based on the optimum ratio established by the above experiment. The reaction time was 3 h with enzyme - substrate ratio of 0.2. The enzyme - substrate ratio of 0.2 was chosen as a result of several trial and errors at which the amount of TSN produced was found to be comparable to that obtained with acid hydrolysis.

PH

Phosphate buffer, pH 7 and 7.5 as well as carbonate buffer, pH 8 - 10 (0.4M) were added to canola meal - enzyme mixture in a proportion of 12:1 (v/w). Temperature and enzyme-substrate ratio were fixed at 66 °C and 0.2 (v/w) respectively in all samples. The hydrolysis was carried out for 3 h.

Reaction time

Enzyme reaction time ranging from 1.3 - 4 h, was tried. Meal - solvent ratio, temperature, pH and enzyme - substrate ratio were fixed at 1:10, 66 C, pH 9 and 0.2 (v/w), respectively.

Enzyme - substrate ratio

Different proportions of enzyme and canola meal (0.1-0.55 v/w) were studied and the hydrolysis was performed under the following conditions: 66 C, pH 9, 1:10 meal - solvent ratio. The canola meal was hydrolysed for 2 h based on the optimum reaction time obtained from the previous experiment.

3.1.1.2 Central composite rotatable design

Three variables, viz., temperature, pH, enzyme - substrate ratio, were further investigated and optimized. However, meal-solvent ratio and the duration of the hydrolysis were fixed at 1:10 (w/v) and 2 h, respectively, since the meal - solvent ratio will be ultimately dictated by the ratio commonly used in sauce production while 2 h appeared to be the maxium reaction time beyond which no further increase in soluble nitrogen was detected.

A response surface experiment of central composite design adapted from Box and Wilson (1951) (Table 3) was chosen for economy in the number of experiments required. The three variables to be optimized were assessed at 5 levels. The levels of the variables were based on the optimum values found in the previous one-factor-at-a-time experiments. The value of $\alpha$ (= 1.682) in this design was obtained from Myers (1976). Table 4 shows different combinations of the coded and uncoded levels of the variables used.

### 3.1.2 Enzymatic Hydrolysis

3.1.2.1 Materials:

a) Defatted canola meal (Western Canadian Seed Processor Ltd., Lethribridge, Alta.)

b) Alcalse 0.6L (Novo Industri A/S, Enzyme Division, Novo Alle, DK - 2880 Dagsvaerd, Demark)

c) Buffer solutions:

pH 7, 7.5, 8, 8.3; 0.4M phosphate buffers prepared as in Sorensen (1909)

pH 8.7, 9.0, 9.4, 9.6, 9.7, 10.0; 0.4M carbonate-bicarbonate buffers prepared as described by Delory et al. (1945)

d) All chemicals used for micro-kjeldahl analysis were of

Table 3. Codings for each level of the variables

| | Code | | | | |
|---|---|---|---|---|---|
| | -1.682 ($-\alpha$) | -1 | 0 | 1 | 1.682 ($\alpha$) |
| Temperature,C (X1) | 61.0 | 63.8 | 68.0 | 72.2 | 75.0 |
| PH (X2) | 8.0 | 8.4 | 9.0 | 9.6 | 10.0 |
| E/S, v/w (X3) | 0.20 | 0.24 | 0.30 | 0.36 | 0.40 |

Table 4. Central Composite Rotatable Design

| Coded | | | Uncoded | | |
|---|---|---|---|---|---|
| X1 | X2 | X3 | Temp. (°C) | PH | E/S(v/w) |
| -1 | -1 | -1 | 63.8 | 8.4 | 0.24 |
| 1 | -1 | -1 | 72.2 | 8.4 | 0.24 |
| -1 | 1 | -1 | 63.8 | 9.6 | 0.24 |
| 1 | 1 | -1 | 72.2 | 9.6 | 0.24 |
| -1 | -1 | 1 | 63.8 | 8.4 | 0.36 |
| 1 | -1 | 1 | 72.2 | 8.4 | 0.36 |
| -1 | 1 | 1 | 63.8 | 9.6 | 0.36 |
| 1 | 1 | 1 | 72.2 | 9.6 | 0.36 |
| -1.682 | 0 | 0 | 61.0 | 9.0 | 0.30 |
| 1.682 | 0 | 0 | 75.0 | 9.0 | 0.30 |
| 0 | -1.682 | 0 | 68.0 | 8.0 | 0.30 |
| 0 | 1.682 | 0 | 68.0 | 10.0 | 0.30 |
| 0 | 0 | -1.682 | 68.0 | 9.0 | 0.20 |
| 0 | 0 | 1.682 | 68.0 | 9.0 | 0.40 |
| 0 | 0 | 0 | 68.0 | 9.0 | 0.30 |
| 0 | 0 | 0 | 68.0 | 9.0 | 0.30 |
| 0 | 0 | 0 | 68.0 | 9.0 | 0.30 |
| 0 | 0 | 0 | 68.0 | 9.0 | 0.30 |
| 0 | 0 | 0 | 68.0 | 9.0 | 0.30 |

analytical grades.

### 3.1.2.2 Equipment

1) Polycarbonate centrifuge tube :

    75 mL capacity with Noryl cap assembly (Nalgene)

2) Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Inc. Edison, NJ)

3) Ultra-centrifuge:

    Beckman model L2-65B Class G with preparative rotor type 21 (Palo Alto, CA)

4) Filter pads:

    Whatman No.1, 7.0 cms diameter (Fisher Scientific)

5) Polyester centrifuge tube:

    250 mL capacity with safty guards and lids (Nalgene).

### 3.1.2.3 Procedure

Specific amount of Alcalase 0.6L as indicated in the experimental design was pipetted into a 75 mL centrifuge tube containing 5g defatted canola meal. Except for the study of meal-solvent ratio, in which 10g defatted canola meal without any enzyme addition were put into 250 mL centrifuge tube. Appropriate amount of buffer solution with corresponding pH or deionized water in the case of meal-solvent ratio experiment was added and the suspension was shaken thoroughly. The mixture was then incubated in the temperature controlled shaker for a certain period of time at a pre-selected temperature as indicated by the experimental design. Enzyme hydrolyzates or canola meal-solvent suspensions obtained after the incubation were centrifuged at 14,000 r.p.m. and the supernatants were filtered under vacuum. Each filtrate was made up to its original volume of added solvent with deionized water. About 0.5 mL aliquots were taken for the analysis of total soluble nitrogen using micro-kjeldahl technique as described by Pearson (1976).

## 3.2 Production of Koji

### 3.2.1 Rehydration of Freeze-dried Mold Culture

3.2.1.1 Materials

a) Mold cultures

   *Aspergillus oryzae* 14895 (NRRL 1989)
   *Aspergillus soja* 16320
   (ATCC, 12301 Parklawn Drive, Rockville, MD)

b) Potato Dextrose Agar (Difco Laboratories, Detroit, MN)

c) Peptone water

3.2.1.2 Equipment

1) Low temperature incubator (Precision, GCA Corporation 3737 West Cortland St. Chicago, IL)

2) Roux bottles

3.2.1.3 Procedure

The tip of the outer vial was heated with bunsen burner. The hot tip was cracked by squirting a few drops of water and then striking with a file. With a pair of sterile tweezers, the fibre glass insulation and the inner vial were removed. The cotton plug in the inner vial was aseptically raised. Sterilized (0.3mL) peptone water was added into the inner vial and mixed to resuspend the lyophilized pellet.

A 0.1 mL portion of the mixture was aseptically transferred onto potato dextrose agar (PDA) slants or plates. The slants or plates were then incubated at 37 C for 48 h. Colonies of similar morphology were subcultured for at least 2 - 3 times.

To harvest the mold culture, 5 mL peptone water was added into the sub-cultured slant tubes. The mold mycelia were scraped and loosened with a spatula. The mold suspension was aseptically transfered into a roux bottle with pre-prepared PDA. The culture was incubated at 37 C for 48 h.

### 3.2.2 Koji Preparation

#### 3.2.2.1 Materials

a) Defatted canola meal, ground and sifted through a 40 mesh sieve. (Western Canadian Seed Processor Ltd., Lethebridge, Alta.)

b) Wheat grains purchased from a local supplier, roasted and ground.

c) Alcalase 0,6L (Novo Industri A/S, Enzyme division, Novo Alle, DK-2880 Dagsvaerd, Denmark)

d) Mold cultures

*Aspergillus oryzae* 14895 (NRRL 1989)
*Aspergillus sojae* 16320
(ATCC, 12301 Parklawn Drive, Rockville, MD)

### 3.2.2.2 Equipment

1) PH-stat (Metrohm AG AH-9100 Herisau, Switzerland)

The combi-titrator 3D used in this research was a combination of a pH-meter E512, Impulsomat E473 and Multi-Dosigraph E425. The Multi-Dosigraph was equipped with a 500 mL double walled jacketted hydrolysis vessel connected to a temperature controlled water bath circulator (Braun Thermomix 1441, B. Braun Melsungen, West Germany). The hydrolysis vessel was covered with a multi-socket lid and fitted with a pH-electrode, an autoburette, a magnetic stirrer and a stir-plate. Admission of 1M NaOH was automatically controlled by the pH-meter, pH-electrode and the impulsomat unit.

2) Temperature controlled water bath (Fisher Scientific)
3) Small mannually operated screw press (purchased from a local drug store).

### 3.2.2.3 Procedure

Fifty grams defatted canola meal was mixed with 210 mL distilled water and 15.4 mL Alcalase 0.6L in the hydrolysis vessel which was connected to the water bath circulator with temperature adjusted to 69 C. An electrode, connected to the Impulsomat, was inserted through the lid socket into the mixture to act as a 'signal messenger' to detect the pH changes during the hydrolysis. Impulsomat was adjusted to a set-point of pH 9 so that NaOH would be automatically fed in to maintain the pH of the mixture, which was constantly stirred with a magnetic stirrer. Volume of NaOH was recorded on the Dosigraph after 2 h of incubation. Appropriate amount of water was then added to the mash to make up the total liquid volume to 250 mL.

The enzyme hydrolyzate was then adjusted to pH 5.5 with concentrated HCl. About 140 mL of liquid was pressed from the mash and kept in a refrigerator for later use. The residue mash was then thoroughly mixed with 50g of roasted wheat and a roux bottle of Aspergillus culture prepared as described in Section 3.2.1.3.

The resulting mixture, known as koji, was analyzed to ensure that its moistuure content was 40-45%. The koji was then incubated at 28 °C for 72 h with occassional stirring.

## 3.3 Moromi Fermentation

The pressed liquid hydrolyzate was mixed with appropriate amount of salt to make up an 18% brine. The brine was then added into the mature (i.e. 72 hour old) koji. A few drops of concentrated lactic acid were added to lower the pH to 5.5, if necessary. The mash (moromi) was left to ferment at room temperature and was stirred three times weekly. A small portion was taken for total soluble solid and pH measurements every 3 - 4 days throughout the fermentation which lasted 5 wk.

## 3.4 Extraction and Pasteurization

### 3.4.1 Equipment

1) Hand press
2) Polycarbonate centrifuge bottles, 250mL capacity (Beckman Instruments Inc., Fullerton, CA)
3) Preparative ultracentrifuge equipped with JA14 rotor and a speed of 10000 r.p.m.. (Beckman Instruments Inc., Fullerton, CA)
4) Temperature controlled water bath with temperature preselected at 75 °C (Fisher Scientific).
5) Filter pads: Whatman No. 1, 7.0 cms diameter (Fisher Scientific)

### 3.4.2 Procedure

Fermented moromi was pressed manually to extract the liquid from the cake. The liquid was pasteurized at 75 C for 30 min in a temperature controlled water bath and was then cooled slowly to allow precipitation of proteinaceous substances and other suspended particles. Further clarification of the turbid liquor was performed by centrifugation at 10,000 r.p.m. for 30 minutes, followed by vacuum filtration. The resultant clear canola sauce was ready to be bottled.

The entire manufacturing process of canola sauce is illustrated in Figure 17.

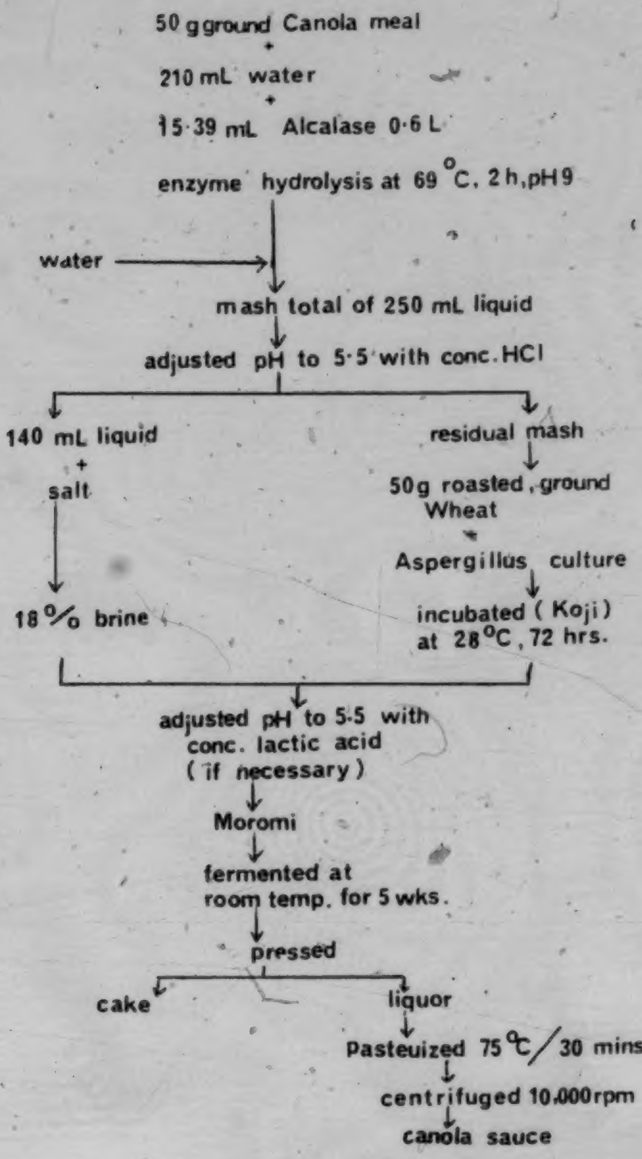

50 gground Canola meal
+
210 mL water
+
15·39 mL Alcalase 0·6 L
enzyme hydrolysis at 69 °C, 2 h, pH 9
water
mash total of 250 mL liquid
adjusted pH to 5·5 with conc. HCl
140 mL liquid
+
salt
18 % brine
residual mash
50g roasted, ground Wheat
+
Aspergillus culture
incubated (Koji) at 28 °C, 72 hrs.
adjusted pH to 5·5 with conc. lactic acid (if necessary)
Moromi
fermented at room temp. for 5 wks.
pressed
cake
liquor
Pasteuized 75 °C / 30 mins.
centrifuged 10,000 rpm
canola sauce

Figure 17 Flow chart of enzymatic production of canola suace

## 3.5 Analysis of Canola Sauce

### 3.5.1 Fractionation of Soluble Components

#### 3.5.1.1 Materials and equipment

a) Cation exchange resin:

Dowex 50w-x8, 100-200 mesh in H+ form (Bio-Rad. Labs. Richmond, CA)

b) Anion exchange resin:

Dowex 1-x8, 100-200 mesh in Cl- form (Bio-Rad. Labs. Richmond, CA)

c) Evaporator: Buchi Rotavapor-R with water bath maintained at 30 °C. (Foss Electric Canada Ltd., 408 Pitt St., Cornwall, Ont.)

Preparation of cation exchange column

Cation exchange resin was washed several times with distilled water and suspended in the starting buffer with pH adjusted to 1.0 by adding 1 M HCl dropwise to water while stirring continuously. Further addition of the acid might be necessary to maintain the pH of the slurry . The resin was then transferred to a glass column (60 x 1.5 cm i.d.) and filled up to 15 cm. To equilibrate the column, the starting buffer was passed through the column slowly until no change in pH was noted in the effluent.

Preparation of anion exchange column

Anion exchange resin, in Cl- form, was first converted to HCOO- form according to the procedure suggested by the Bio-Rad ion exchange manual. The resin was equilibrated with

0.5M NaOH in the similar manner as described above.

### 3.5.1.2 Procedure

Canola sauce sample was diluted 20 times with distilled water. Ten mL aliquot of the diluted sample was adjusted to pH 1.5 - 2.0 with 1M HCl and carefully poured into the cation exchange column. The sample was allowed to penetrated the column slowly until the liquid was level with the top of the resin. The column was then washed with 70 mL water at a rate of approximately 1 drop per second. Seventy mL effluent was collected and further subjected to the anion exchange column. Again, the column was washed with 70 mL water and another 140 mL effleuent was collected. This effluent was evaporated and reconstituted with 5 mL distilled water. This fraction contained neutral compounds such as sugars and was 10 times less concentrated than the original sauce.

The cation exchange column was eluted with 140 mL of 4M $NH_4OH$, rapidly in the first 30 mL and then slowly at about 1 drop per second for the last 110 mL. The euent was evaporated to dryness until free of ammonia. To the residue was added a few drops of HCOOH and re-evaporated. The residue was reconstituted with 5 mL distilled water. This fraction consisted of 10 times diluted amino acids and other nitrogenous compounds as present in the sauce.

The anion exchange column was eluted with 80 mL of 4M HCOOH with flow rate adjusted to about 1 drop per second. The eluent was collected and evaporated to dryness and free of pungent odor. The residue was redissolved in 5 mL water.

This fraction represented 10 times diluted organic acids as found in the sauce sample.

The fractionation procedure was illustrated in the flow-chart (Figure 18).

To regenerate the cation exchange column, 70 mL of 2M HCl was used to wash the resins. Both columns were equilibrated (see 3.5.1.2.) before the next sample was applied.

### Elution efficiency test of cation exchange chromatography

Elution efficiency of the cation exchange chromatography was tested indirectly in this experiment. Since sauce samples prepared varied in their amino nitrogen contents, 10 mL aliquot of 20 times diluted commercial soy sacue was chosen as a standard for the efficiency test. Ten mL fractions of eluate after ammonium hydroxide elution from the column were collected, evaporated and redissolved in water as described in the procedure. The reconstituted fraction was then tested for the presence of amino nitrogen with ninhydrin. Absence of amino nitrogen was found at the twelfth fraction (i.e. 120 mL eluent). To assume complete elution of sample, an addittional 20 mL of ammonium hydroxide were used to wash the column, bringing the total quantity of the eluent to 140 mL.

### Elution efficiency test of anion exchange chromatography

Elution efficiency of anion exchange chromatography was tested in the same manner as that in the cation exchange column , i.e., 10 mL fractions of eluate after HCOOH elution

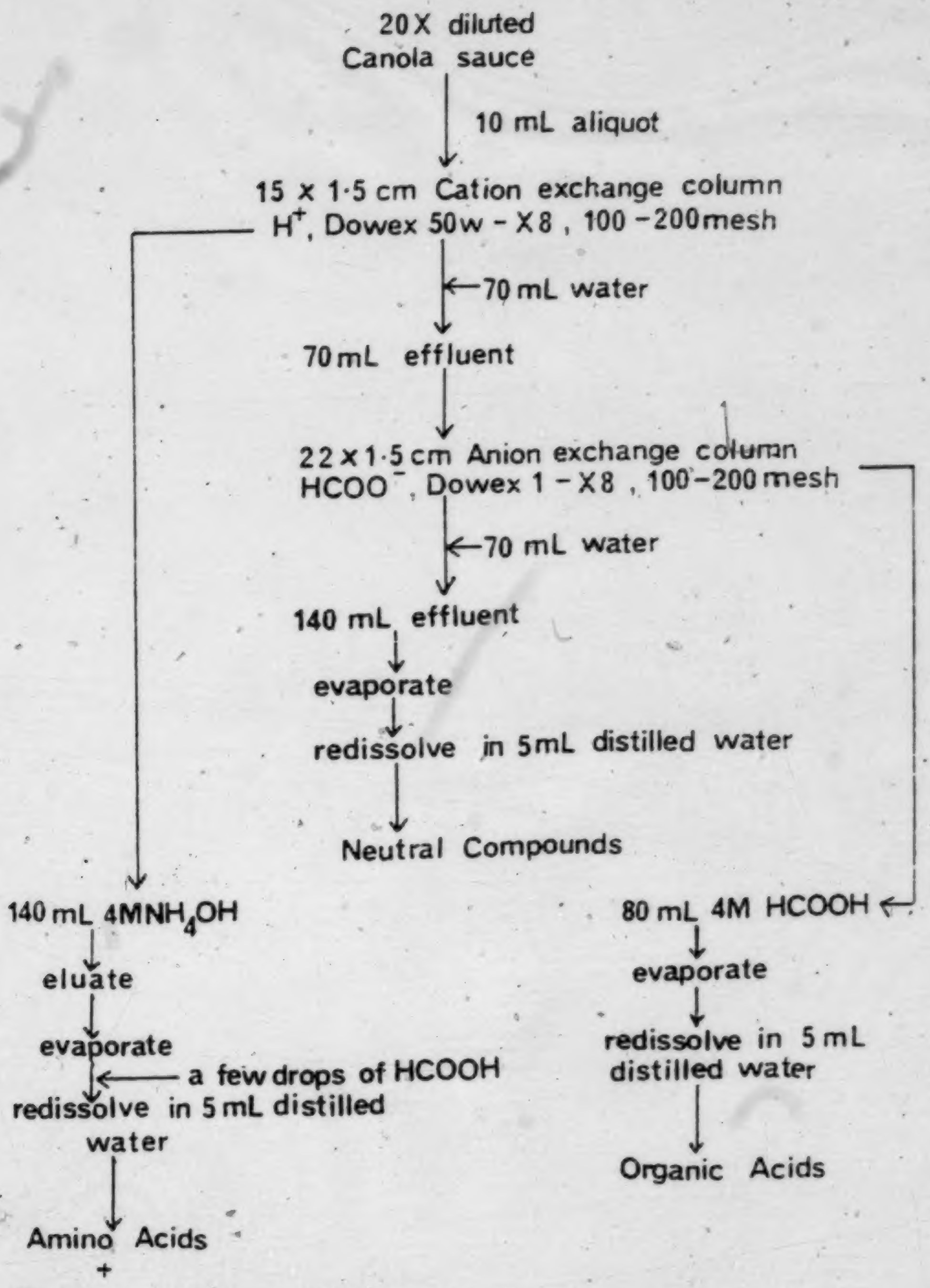

20X diluted
Canola sauce
10 mL aliquot
15 x 1·5 cm Cation exchange column
H+, Dowex 50w - X8, 100 -200mesh
70 mL water
70 mL effluent
22 x 1·5 cm Anion exchange column
HCOO−, Dowex 1 - X8, 100-200 mesh
70 mL water
140 mL effluent
evaporate
redissolve in 5mL distilled water
Neutral Compounds
140 mL 4MNH4OH
eluate
evaporate
a few drops of HCOOH
redissolve in 5 mL distilled water
Amino Acids
+
Nitrogenous Compounds
80 mL 4M HCOOH
evaporate
redissolve in 5 mL distilled water
Organic Acids

Figure 18 Fractionation of soluble components in canola sauce.

were collected and injected into the HPLC unit used for organic acid analysis. Absence of organic acid was found at the seventh fraction (i.e., after the additon of 70 mL eluent ). To assume complete elution of sample, an additional 10 mL of HCOOH was used to wash the column bringing the total quantity of the eluent to 80 mL.

### 3.5.2 Analysis of Nitrogen Containing Compounds

#### 3.5.2.1 Quantitation of amino nitrogen

One mL aliquot of the cation - exchanged fraction was diluted 10 times with deionized water. 0.5 mL of this diluted sample was reacted with ninhydrin solution according to Rosen (1957). The resulting sample-ninhydrin solution was further diluted by adding 20 mL of isopropyl alcohol-water diluent. Amino nitrogen content of the sample was measured colorimetrically at 570 nm and the value in mMoles leucine equivalent/100 mL sauce of amino nitrogen was obtained from a standard curve.

The standard curve was prepared by using leucine as the amino nitrogen source and 20 mL isopropyl alcohol-water as diluent (Figure 19).

#### 3.5.2.2 Quantitation of total soluble nitrogen

Micro-kjeldahl analysis adapted from Pearson (1976) was used to quantitate total soluble nitrogen of the sauce. The sample size used was less than 0.5 mL.

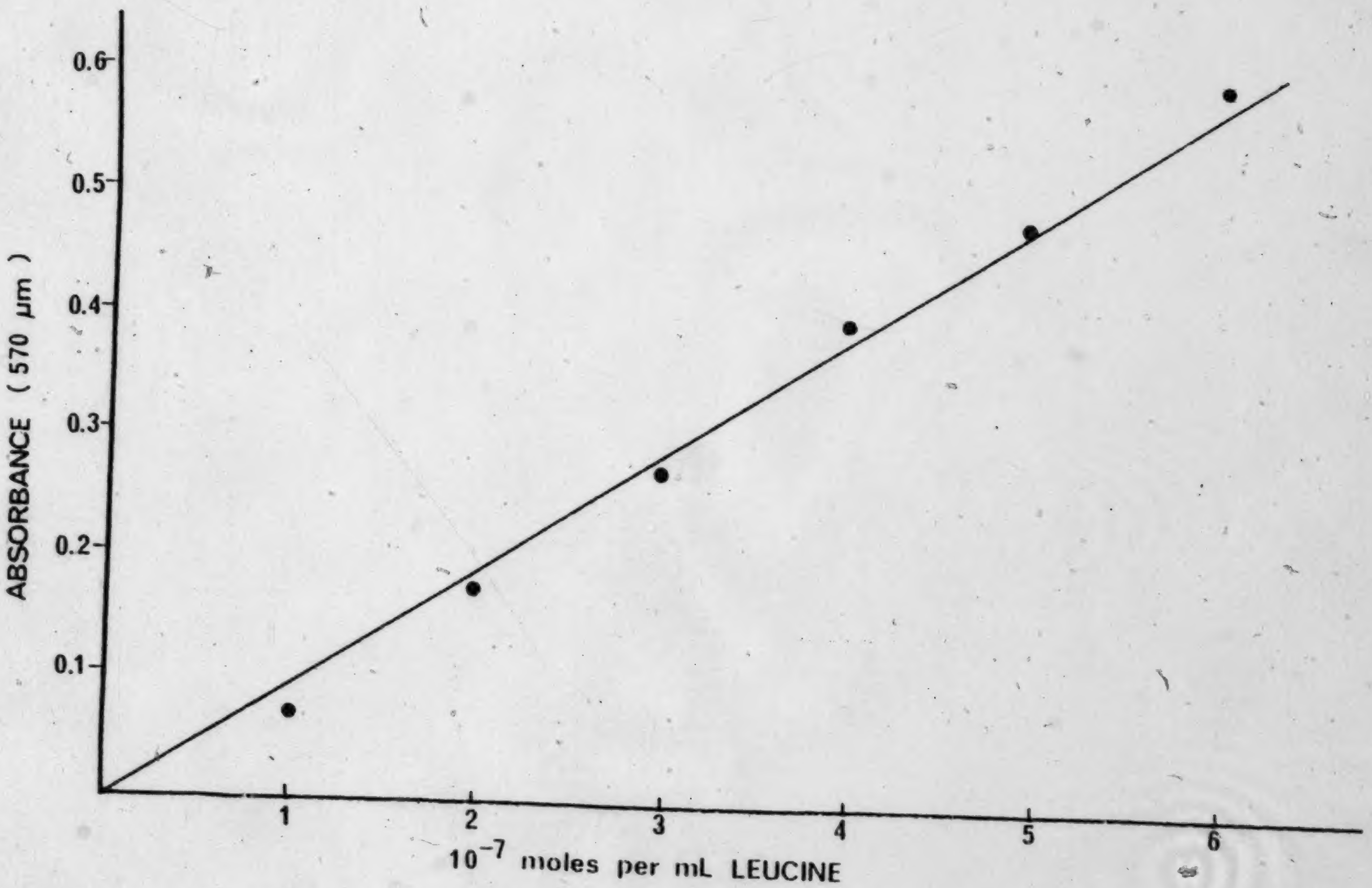


Figure 19 Calibration curve of ninhydrin - leucine color yield.

### 3.5.2.3 Analysis of amino acids by means of Automatic Amino Acid Analyzer

One mL cation exchange eluent was first diluted 5 times with HCl (pH 2.2). The sample was transferred into a storage coil using a syringe. The sample storage coils were then placed on the turntable of the automatic sample injector. Analysis of amino acids was performed automatically in a Beckman Amino Acid Analyzer Model 121 MB (Beckman Instruments, Inc., Palo Alto, CA). Three buffer solutions of pH 3.28, 3.90 and 4.95 with flow rate adjusted to 10 mL/h were employed to elute the amino acids present in the sample through the AA-10 column with temperature programmed to 50 - 65 C. Amino acids after the elution were reacted with ninhydrin reagent (Flow rate at 5 mL/h) in a heated reaction bath. Color intensity of the amino acid - ninhydrin complex was detected by a colorimeter installed in the same unit. Caoncentrations of each amino acid in the sample was calculated and recorded in a Beckman Model 126 Data System.

The operating procedures and reagents required for the analysis were described in the Users' manual provided by the company. All standard buffers and reagents used for the analysis were purchased from the same company.

## 3.5.3 Analysis of Acidic Compounds

### 3.5.3.1 Quantitation of total titratable acidity

Total acidity of the sauce was determined by titrating 1 mL aliquot of the anion - exchanged eluent with 0.005M

NaOH using phenolphthalin as an indicator.

### 3.5.3.2 HPLC Analysis of Organic Acids

Materials

a) citric acid - anhydrous (Sigma)
b) iso - citric acid - 93-98% crystalline (Sigma)
c) pyruvic acid - 99% (Sigma).
d) malic acid - 98-100% crystalline (Sigma)
e) trans - aconitic acid - 98-100% (Sigma)
f) glyoxylic acid - 98% (Sigma)
g) succinic acid - 99% (Terochem)
h) formic acid - 97% (Aldrich)
i) lactic acid - 88% analytical grade (Terochem)
j) propionic acid - 99% (Sigma)

Equipment

1) HPLC unit

The HPLC unit was manufactured by Bio-Rad Laboratory, Mississauga, Ont.. This unit consisted of a Model 1330 HPLC pump, a strong cation exchange column, 300 x 7.8 mm, packed with Aminex HPX - 87 resin, a micro-guard HPLC column, a model 1305 ultraviolet spectrophotmetric detector with a standard deuterium lamp, and a flatbed chart recorder model 1321. A model 7125 syringe sample injector (Rheodyne Inc., CA) was used for constant 20uL of sample loading.

2) Centrifuge IEC Model HN-SII (Damon/IEC Division,

International Equipment Division, Needham Heights, MA)

Procedure

Organic acid fraction obtained after anion exchange chromatography was decolorized by shaking with a small amount of charcoal, followed by centrifugation before it was analyzed. The following conditions were employed in the analysis of organic acids by HPLC:

- the mobile phase was 0.012N $H_2SO_4$ with flow rate at 0.8mL/min.
- column temperature was pre-set at 65°C
- the ultraviolet detector was monitored at 210nm with attenuation of 0.02 or 0.04.

Each sample was analyzed at the attentuation of 0.02 and 0.04 and the results were recorded on a chart recorder.

Identification of sample components was carried out by comparing their retention times with those of the pure standards. These were further confirmed by using ' spiking ' technique which involved an addition of known standard organic acids into the sample, one at a time, and identifying the peaks that increased in height.

Quantitation of organic acids in the samples was performed by comparing peak heights of the sample with the calibration curves (Figures 20 - 24). Amounts of standard organic acids (w/v) used in the calibration curve were based on the amounts of those found in the commercial soy sauce by Ueda et al. (1958).

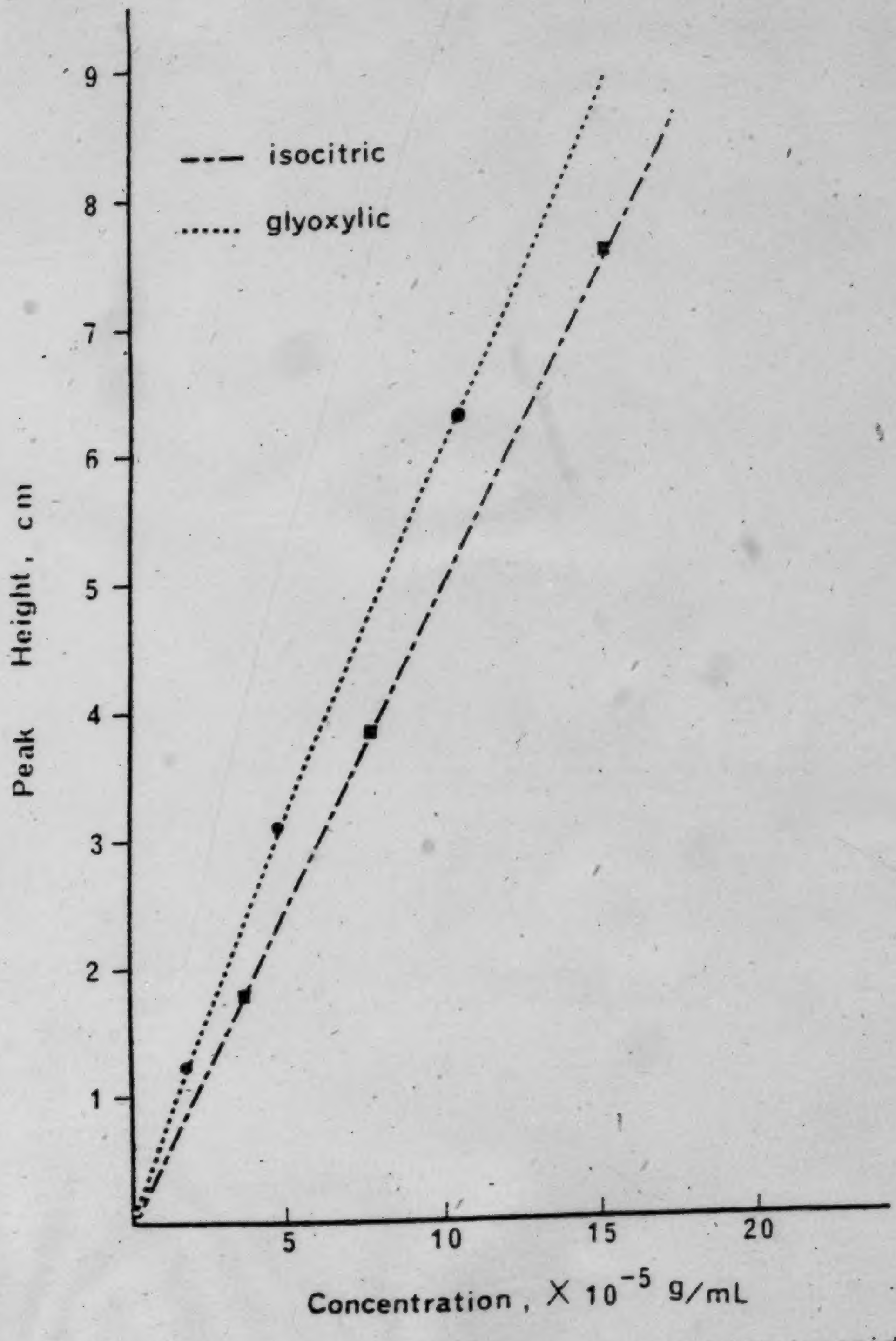


Figure 20 Calibration curves of standard isocitric acid and glyoxylic acid.

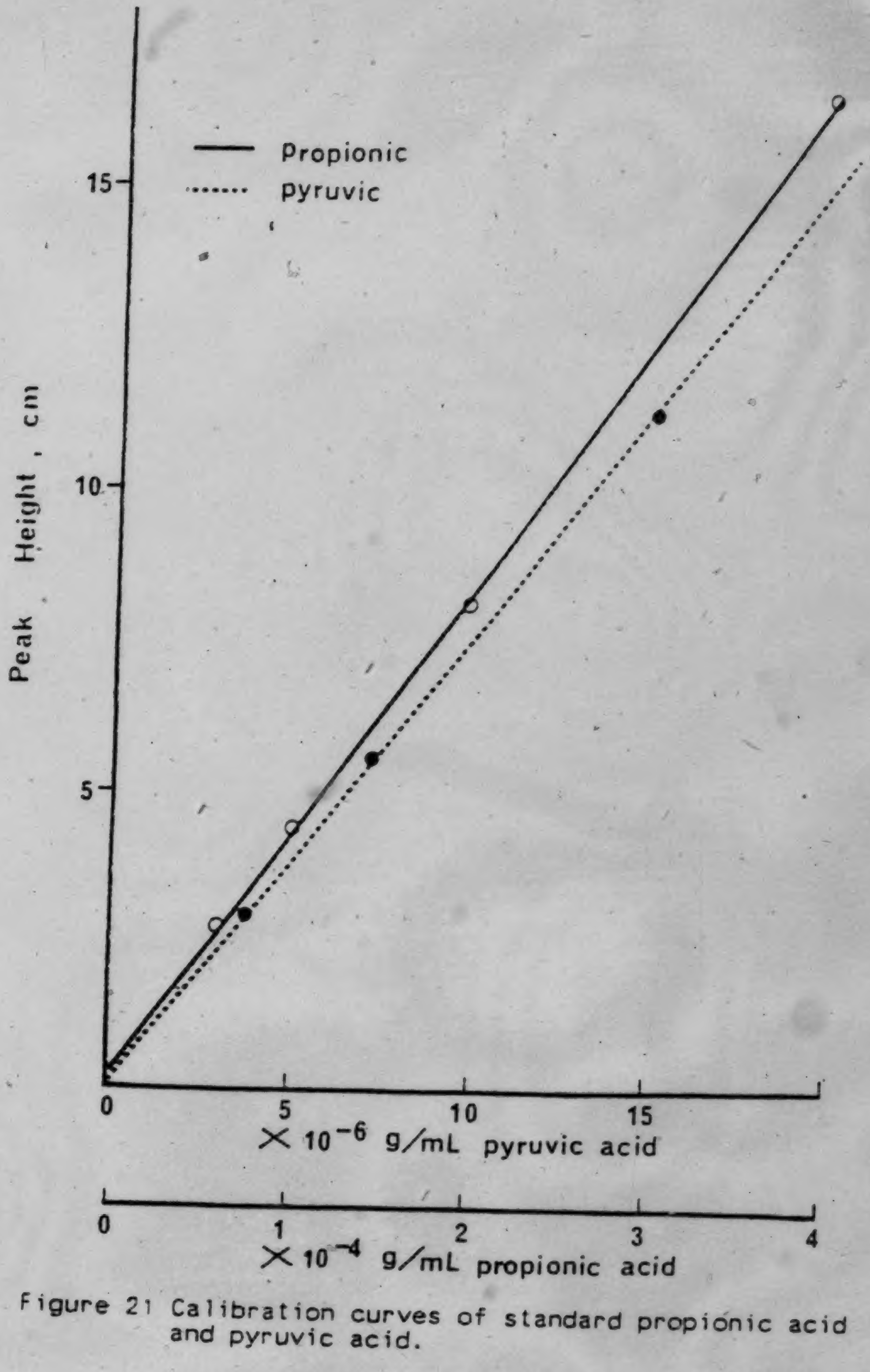


Figure 21 Calibration curves of standard propionic acid and pyruvic acid.

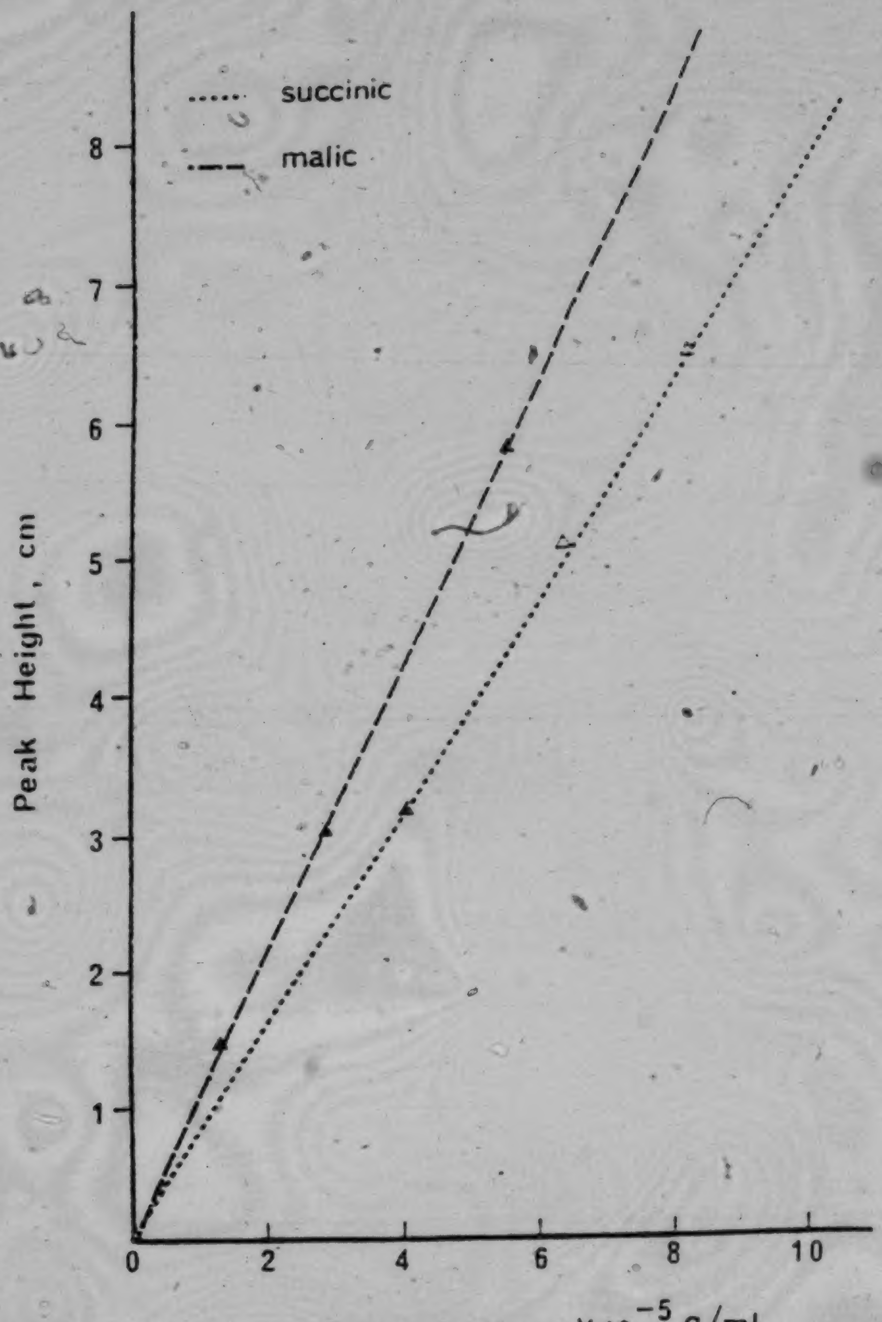


Figure 22 Calibration curves of standard succinic acid and malic acid.

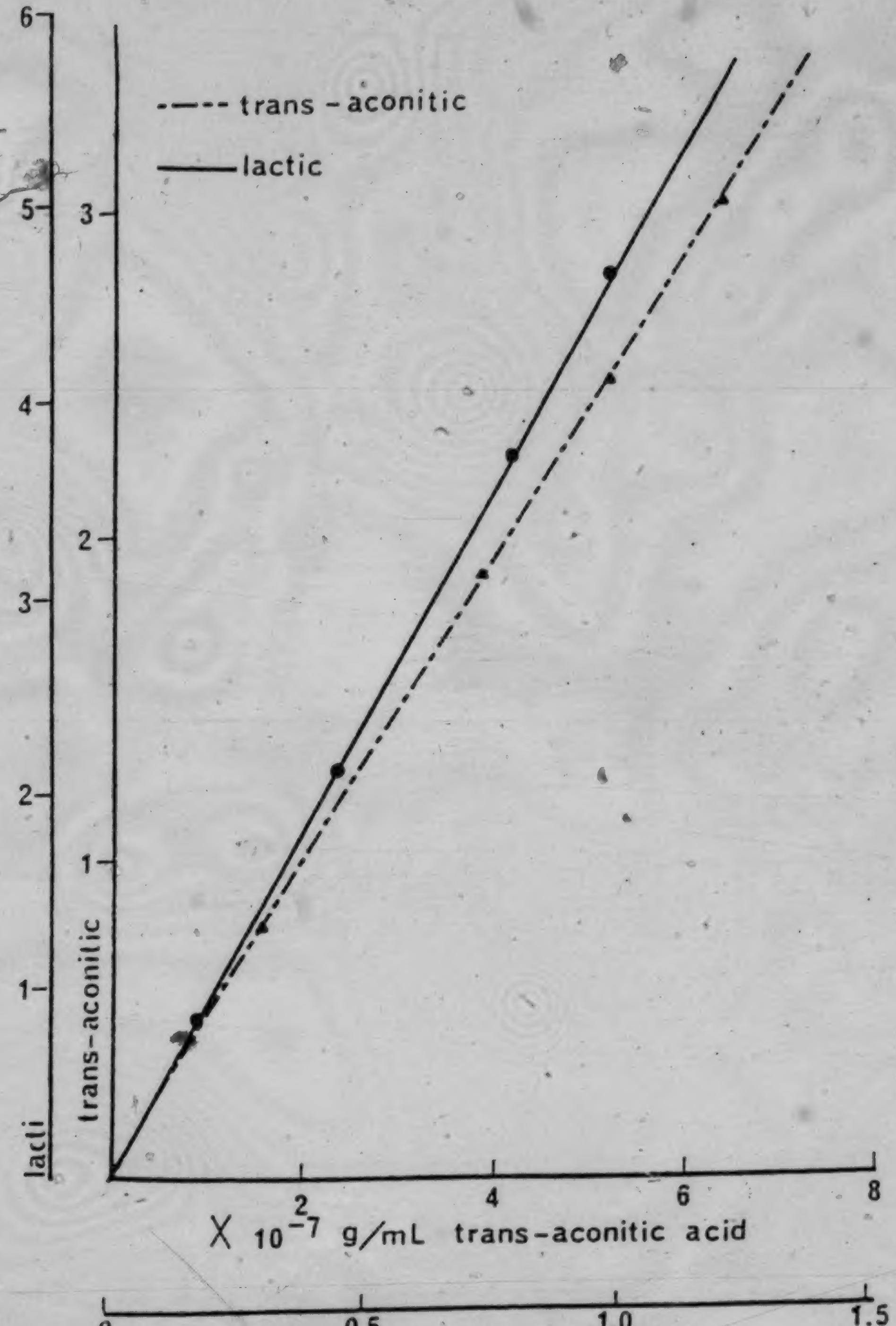


Figure 23 Calibration curves of standard lactic acid and trans-aconitic acid .

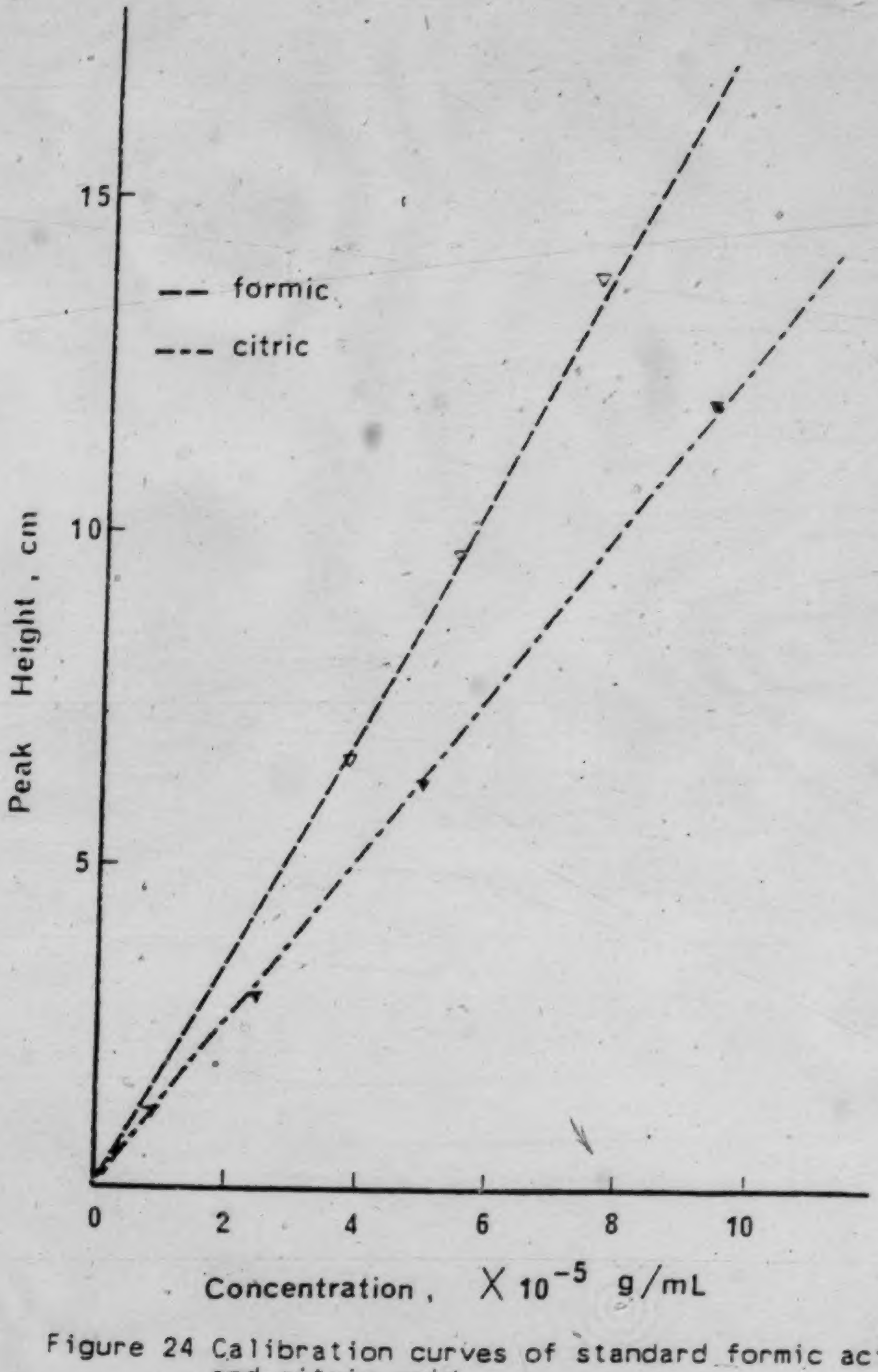


Figure 24 Calibration curves of standard formic acid and citric acid.

### 3.5.4 Analysis of Sugars

#### 3.5.4.1 Determination of reducing sugar

One mL aliquot of the neutral fraction after the ion exchange chromatography was reacted with 25 mL each of Fehling solutions in a 400mL beaker. Water was added to make a final volume of 100 mL. The solution was boiled for 6 minutes. Amount of cuprous oxide produced, equivalent to the amount of reducing sugars, was collected and quantitated by titration with sodium thiosulfate. Weight of invert sugar equivalent was obtained by comparing the result with the weight of copper in the Munson and Walker table. Concentration of reducing sugars was reported as percentage of invert sugar in the sauce sample. (A.O.A.C. 1975, Section 31.038, 31.040, 31.041 and 52.018).

#### 3.5.4.2 Determination of non-reducing sugar

Sample inversion

One mL of neutral fraction was pipetted into a 50 mL volumetric flask containing 20 mL of water. Ten mL of 6.34M HCl was added slowly, while rotating the flask. The flask was placed in a water bath with temperature set at 60°C. The solution was agitated continuously for about 3 min. and left in the bath for exactly 7 min. longer. After the inversion, the flask was placed at once in water at 20 °C until the content was cooled to room temperature. The invert solution was neutralized with NaOH and diluted to the 50mL mark(A.O.A.C. 1975, Section 31.026).

Analytical procedure

Twenty five mL each of copper sulfate and alkaline tartrate solutions was transferred to a 400mL beaker. The invert solution was mixed with the reagents and analysed for reducing sugar content as described above.

Reducing sugars were determined as invert sugar using the Munson and Walker table. Percentage invert sugar obtained in Section 3.5.4.1 was subtracted from that obtained in this experiment. The difference was multiplied by 0.95 to obtain %sucrose. Amount of non-reducing sugar present in the sauce sample was expressed as %sucrose in the sauce.

### 3.5.5 Total Soluble Solids Measurements

Small amount of moromi samples were pressed and total soluble solids (TSS) in the liquor was measured once every 3 -4 days throughout the entire fermentation process. The TSS in moromi and sauce samples were measured with Abbe refractometer (Carl Zeiss, West Germany).

### 3.5.6 PH Measurements

PH of samples were measured with an expanded scale Fisher pH meter.

### 3.5.7 Sodium Chloride Determination

Sodium chloride was expressed in terms of %chloride in the sauce. This was measured in terms of electrical potential (mV) at 25 C with a Fisher Accumet Selective Ion Analyzer Model 1750 (Fisher Scientific Company, Pittsburgh, PA), and a chloride electrode model 94-17 (Orion Research Inc., Cambridge, MA).

The dip-cell and the instrument was calibratied with a KCl standard solution, supplied by the manufacturer, according to the instrument's operation manual.

Calibration curve prepared with standard solutions of NaCl was used to convert readings of 100X diluted samples to %NaCl (Figure 25). Each sample was analyzed in triplicate.

### 3.5.8 Color Measurement

Color of the sauce was measured by means of the Hunter L,a,b Colorimeter model D25L-2 and detector model D25 Optical Head (Hunterlab, 9529 Lee Highway, Fairfax, VA). The instrument was first standardized with different colored ceramic tiles according to the users' mannual supplied by the company. Twenty mL of sample were poured into a petri dish and L, a, b readings were recorded.

### 3.5.9 Moisture Content Determination

About 0.5 g of koji sample was put on a piece of weighting paper and was put in an Automatic Volatility Computer model AVC-MP (CEM Corp., Indian Trail, NC) for 12 minutes. Water content of the sample was determined and recorded automatically by the instrument.

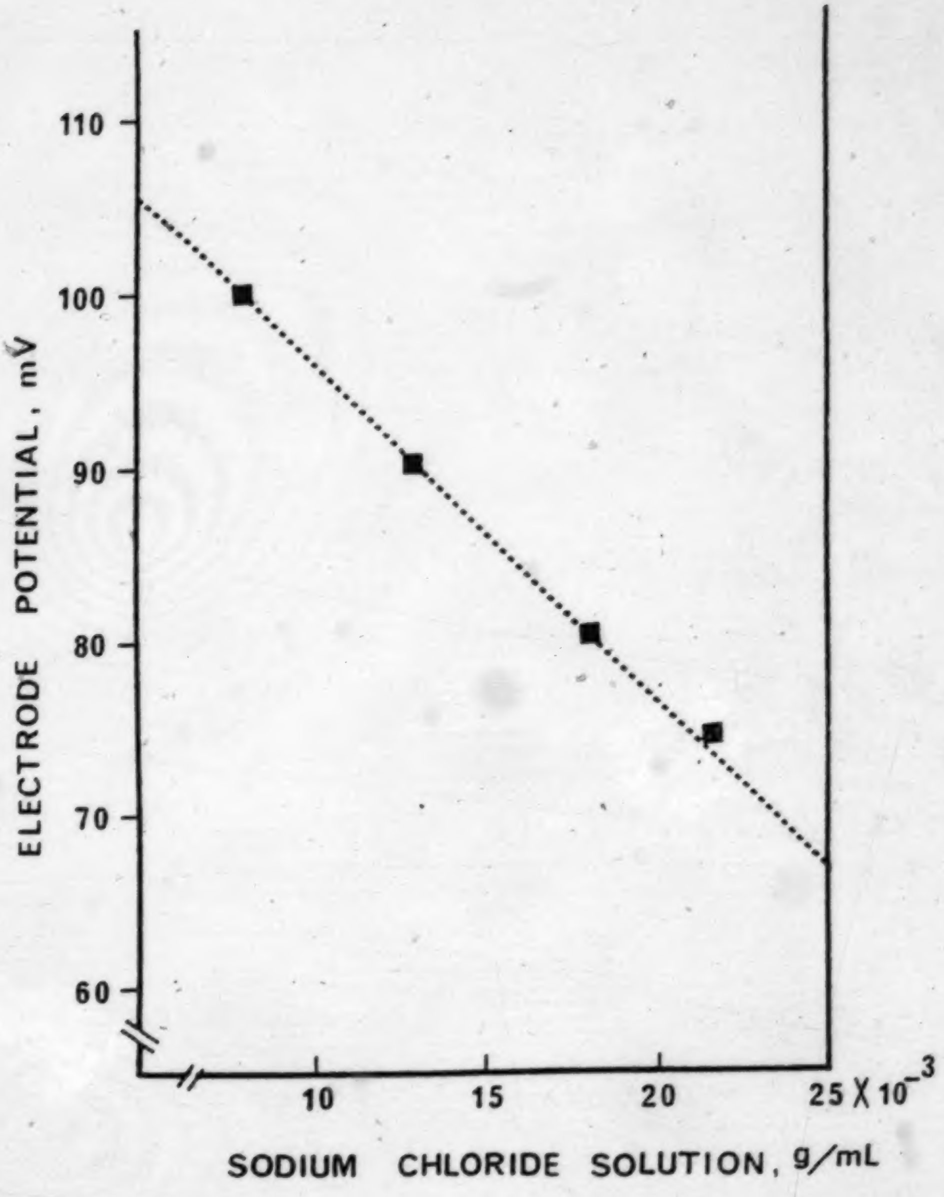


Figure 25 Calibration curve of sodium chloride solution

### 3.6 Sensory Evaluation

Three sensory evaluation sessions were conducted to compare the 5 canola sauce samples, produced from enzymatically prehydrolyzed canola meal , with three commercial soy sauce samples purchased from a local store (Kikkoman shoyu, Chinese soy sauce and China Lily).

The panelists for all 3 sessions consisted of 10 staff and students in the Department of Food Science, the University of Alberta. The samples were scored on 9 point scale, 1 being the least preferred and 9 the most preferred. Three attributes of the sauce were studied viz., aroma, taste and overall acceptance. Individuals were also asked to comment on the samples. The results were statistically evaluated with analyses of variance and Duncan's Multiple Range Test.

The first session was to compare the following samples : (1) canola sauce (CS), fermented for 5 weeks with *A.oryzae*; the moromi was adjusted to pH 5.5 before the fermentation; (2) CS as (1) but with *A.sojae*; (3) as (1) but with the mixture of *A.oryzae* and *A.sojae*; (4) as (1) but the moromi was adjusted to pH 6.5 at the beginning of the moromi fermentation; (6) commercial Kikkoman soy sauce.

The second and third sessions were to compare the 5 canola sauces (sample (1) to (5)) in the same manner as in the first session but sample (6) was replaced with commercial Chinese soy sauce and China Lily, a chemical soy sauce, respectively.

# 4. RESULTS AND DISCUSSION

## 4.1 Optimization of Enzymatic Hydrolysis on Canola-meal

The enzyme used in this research was Alcalase 0.6L, a proteolytic enzyme prepared commercially by submerged fermentation of a selected strain of *Bacillus licheniformis* , purchased form Novo Industri A/S (Novo Alle, DK-2880, Bagsvaerd, Denmark.) It consisted mainly of Subtilisin A (subtilisin Carlsberg) which was an endoprotease of serine type. It hydrolyzed almost all kinds of protein, especially plant proteins, producing soluble low molecular weight peptides. Lacroix et al. (1983) found that low molecular weight peptides liberated by the enzymatic hydrolysis of rapeseed protein improved nutritive value of the product as well.

Attaining maximum solubilization of protein in the substrate was a desirable aim in protein hydrolysis, especially in sauce production where the sauce was essentially a liquid protein hydrolyzate. Therefore, hydrolytic conditions such as temperature, pH, enzyme concentration, reaction time etc., must be determined so that maxium enzymatic activity could be achieved.

Technical data on Alcalase 0.6L provided best conditions as 60 °C, pH 8.5, enzyme concentration between 0.02 - 0.3 AU/L and reaction time of 10 min. The measurements were based on a modified Anson method in which pure hemoglobin was digested by the enzyme to liberate TCA soluble product which could be measured colorimetrically as milliequivalent of tyrosine. Enzyme activity was reported as degree of hydrolysis (DH) or %peptide bonds cleaved (Novo Industri A/S, 1978).

Canola meal, on the other hand, is a complex heterogeneous system in which enzymatic hydrolysis would involve many biochemical and chemical reactions. Therefore, the use of DH to monitor enzymatic activity in such a system might be inadequate or unsuitable.

To avoid getting too involved in the attempt to accurately describe Alcalase activity in canola meal hydrolysis, however, it was thought best to use an indicator which could be simply measured yet adequately reflected the extent of hydrolysis. Total soluble nitrogen (TSN) in the hydrolyzate appeared to meet these requirements; therefore, it was chosen as an index of Alcalase activity in the experiments to optimize hydrolytic conditions.

One-factor-at-a-time technique in which each factor controlling hydrolytic process was individually studied by varying its levels while keeping other factors constant was first used in the optimization experiments. This was to give a coarse indication of optium values for these factors which could then be ' fine-tuned ' with a more sophisticated technique such as response surface methodology (RSM) where all factors were varied simultanously. Results of the experiments are shown in Figures 26, 27 and 29 to 31.

In the temperature experiment where pH, meal-solvent ratio, enzyme-substrate ratio and reaction time were fixed at 8.8, 1:10 (w/v), 0.2 (v/w) and 3 h, respectively, sharp increase in total soluble nitrogen was observed between 60 66 C (Figure 26). In the pH study, maxium TSN was obtained at pH 9 (Figure 27). Note that 1:12 meal-solvent ratio was used in the investigation of the pH-effect while 1:10 was used in other experiments. Adequate solvent, which was buffer solution, must be used to maintain the pH of the system as a-amino groups and free carboxylic groups were liberated during the enzymatic cleavage. In the pH study, the hydrolysis took place mainly under alkaline condition (pH 8 - 10) which was above the average pI value of the amino acids (pK = 6.0 at 25 C). (Figure 28). Therefore, a greater quantity of buffer solutions was required in this case in order to maintain pH at the chosen level. Higher %TSN was found in the pH curve as compared to the results shown on the other curves as also indicated in the meal-solvent ratio curve (Figure 29).

Figure 30 illustrates the effect of duration of the hydrolysis on TSN yield. Highest %TSN was found after the hydrolysis had proceed for 2 h, followed by a substantial decrease. It was noted that the canola meal consisted of an abundant amount of reducing sugars, and the incubation condition (66 C and pH 9) were quite suitable for Maillard reaction to take place. The abrupt decrease in %TSN might,

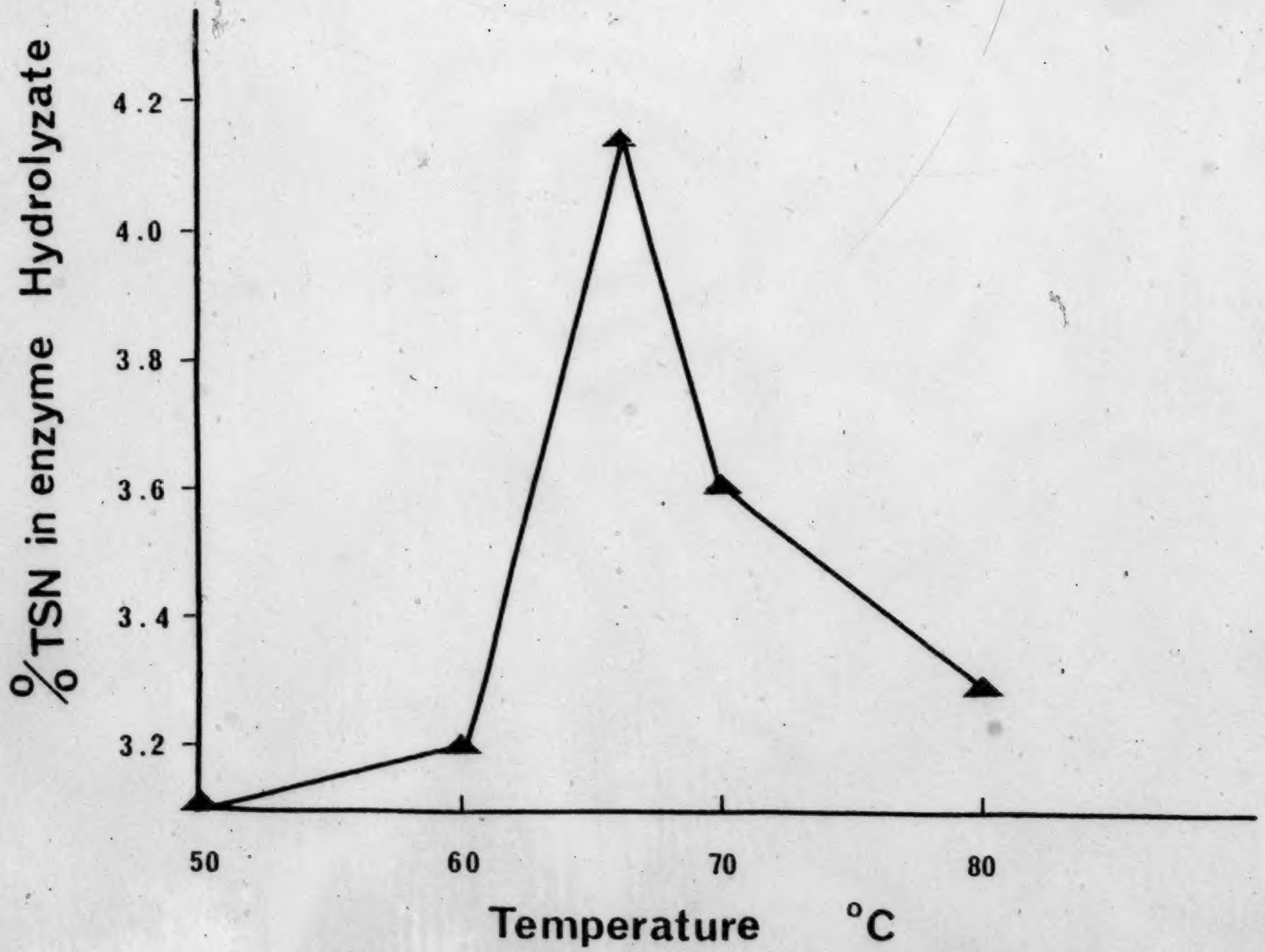


Figure 26 Effect of temperature on percentage total soluble nitrogen yield during enzymatic hydrolysis

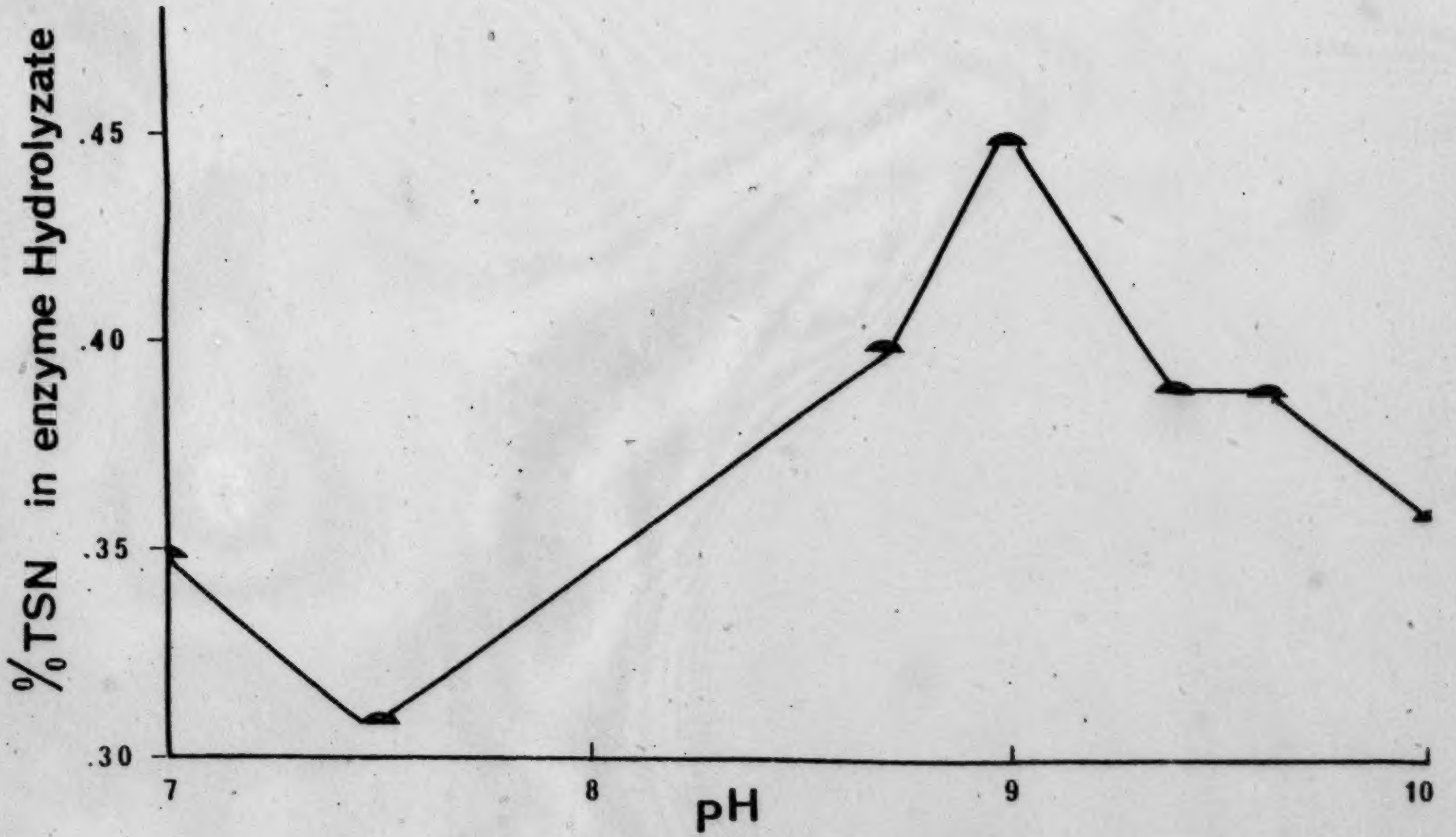
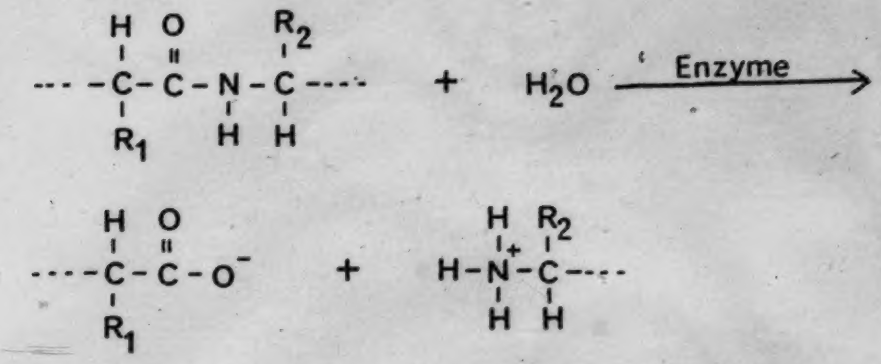


Figure 27 Effect of pH on percentage total soluble nitrogen yield during enzymatic hydrolysis



$$\text{---}\overset{H}{\underset{R_1}{C}}-\overset{O}{\overset{\|}{C}}-\underset{H}{N}-\overset{R_2}{\underset{H}{C}}\text{---} + H_2O \xrightarrow{\text{Enzyme}}$$

$$\text{---}\overset{H}{\underset{R_1}{C}}-\overset{O}{\overset{\|}{C}}-O^- + H-\overset{H}{\underset{H}{\overset{+}{N}}}-\overset{R_2}{\underset{H}{C}}\text{---}$$

The average pI value of amino acids is about 6.0

Figure 28 Enzymatic hydrolysis on protein

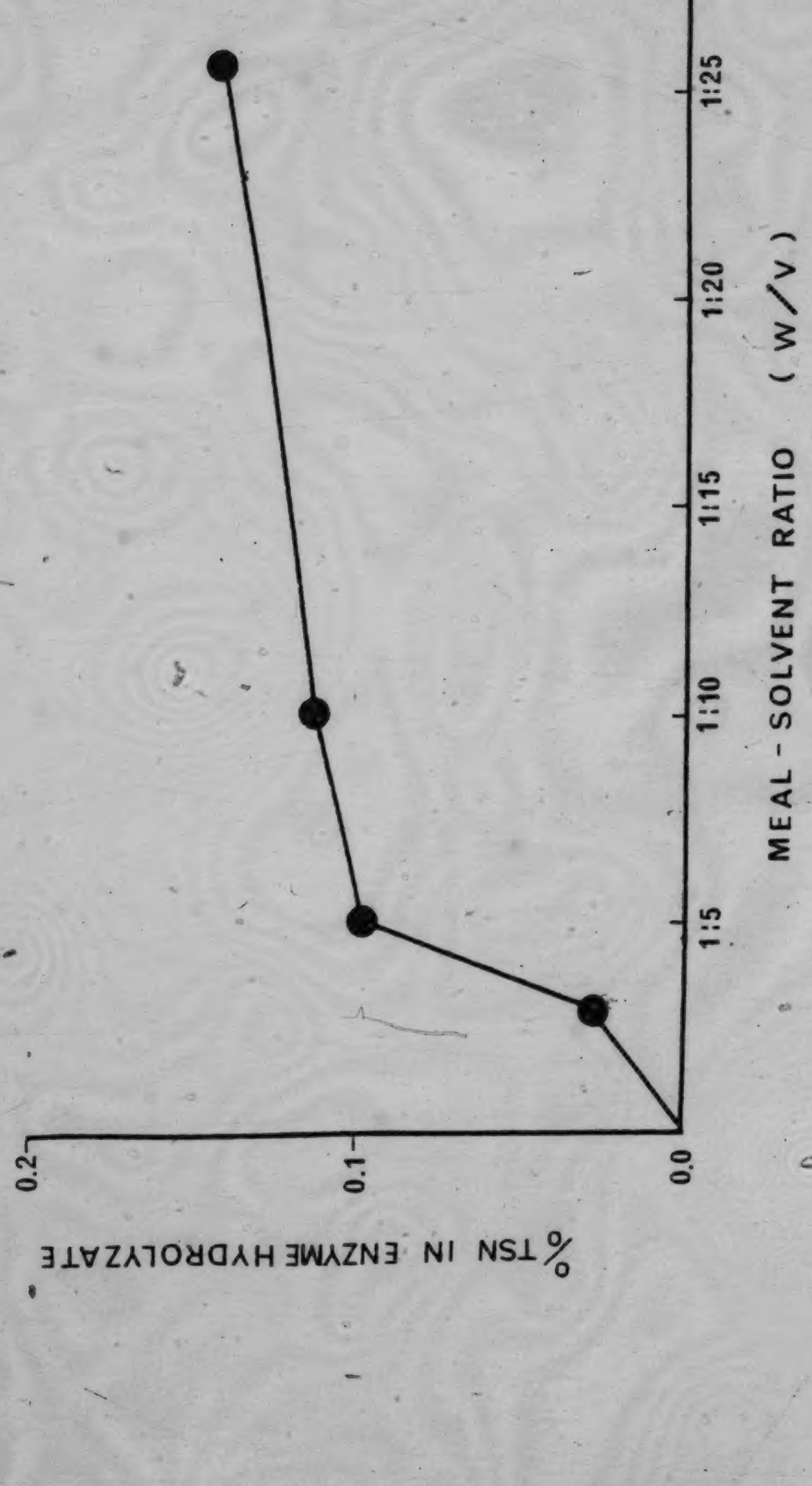


Figure 29 Effect of meal - solvent ratio on percentage total soluble nitrogen yield during enzymatic hydrolysis

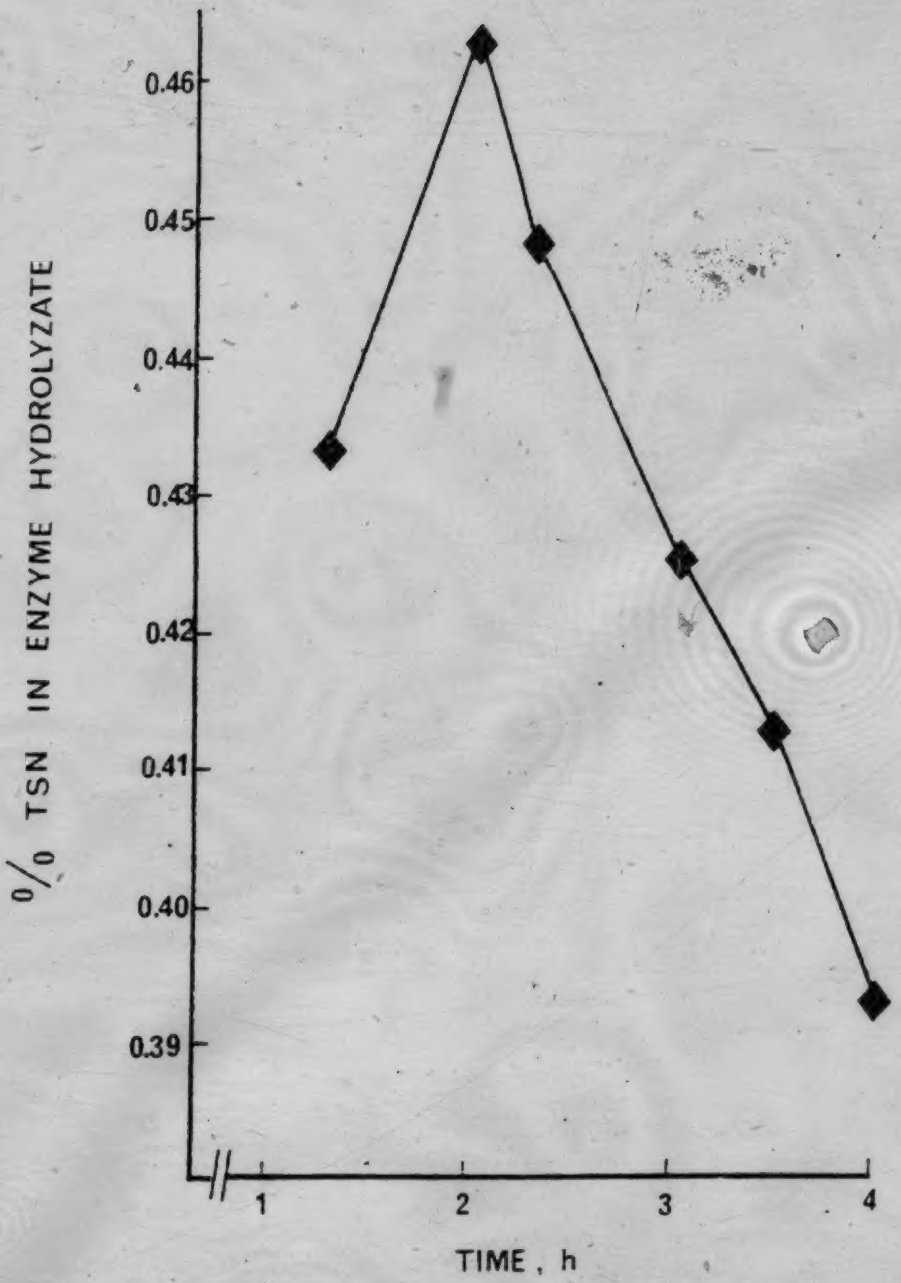


Figure 30 Effect of duration of hydrolysis on percentage total soluble nitrogen yield during enzymatic hydrolysis.

therefore, be attributed to the fact that part of the TSN reacted with reducing sugars in the mash. The resultant amino-carbonyl might subsequently condense and polymerize with each other, leading to the production of color pigments or melanoidin compounds. Such an assumption was, indeed, supported by the fact that the mashes after enzymatic hydrolysis appeared darker than those before the incubation. Furthermore, reaggregation of soluble proteins to insoluble complexes might also occur. Together, they might account for the drastic decrease in TSN in the mash after 2 h.

It is interesting to note that there was no specific trend of enzymatic activity, as measured by %TSN, to indicate the effect of enzyme-substrate ratio (Figure 31). The results appeared to deviate from the general rule of enzyme kinetics. Nevertheless, it is possible that in a complex system such as canola meal, many factor may influence the TSN yield. For example, high cellulose fiber content in canola may prevent easy access of the enzyme to the substrate, and reactions between parts of proteins and other plant materials present in the meal might make the hydrolysis more difficult. It is also possible that because of high substrate concentration, the activity of the enzyme might already have reached the plateau, and further increase in enzymatic concentration would result in its decline.

Again, it should be emphasized that the term 'one-factor-at-a-time' did not truly reflect the actual changes in environment when all factors were present

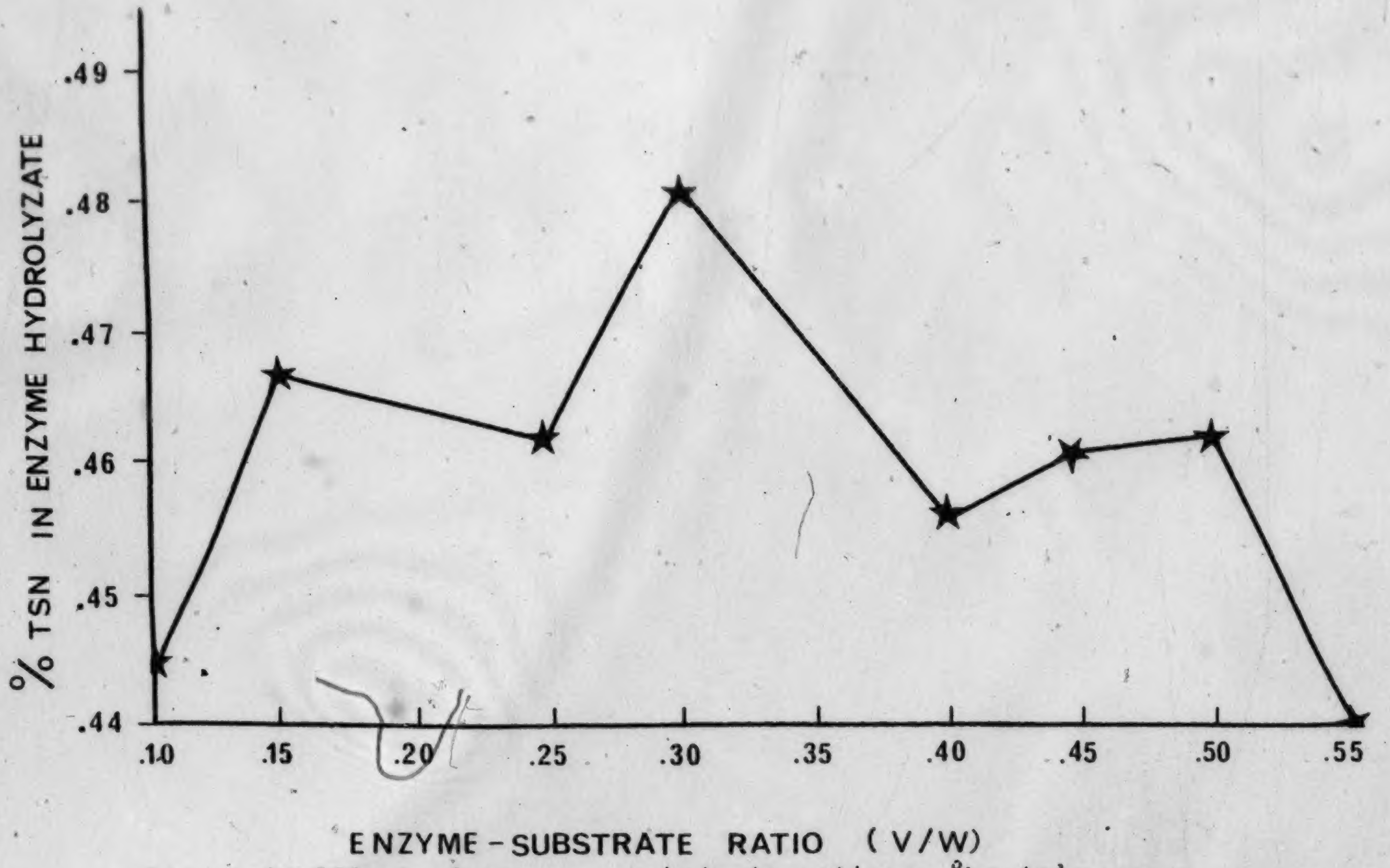


Figure 31 Effect of enzyme - substrate ratio on % total soluble nitrogen yield during enzymatic hydrolysis.

simultaneously. Since it was impossible to investigate all factors involved in a complex substrate system like canola, only a few known rate controlling factors were chosen. Therefore, these preliminary experiments only served as an initial probe to indicate the regions in which the chosen factors should be more fully evaluated. Further evaluation could then be done with a more sophistcated experimental design such as the response surface methodology (RSM) to pinpoint the optimum values of the factors for the process more accurately.

Due to the experimental and processing limitations, meal-solvent ratio and the duration for the hydrolysis were fixed at 1:10 (w/v) and 2 h respectively. The remaining three parameters - temperature, pH and enzyme-substrate ratio were the only factors subjected to further optimization with the RSM.

Meal-solvent ratio was fixed at 1:10 (w/v) due to the fact that there was no significant increase in extractable soluble nitrogen beyond this value (Figure 29). Moreover, the amount of water added to the mash was limited by the sauce concentration. Too diluted a sauce would, of course, fall short in its organoleptic characteristic, while too high a meal - solvent ratio would hamper protein extraction as well as increase the production cost of the sauce. Therefore, a compromise between maximal protein extraction and acceptable sauce concentration had to be achieved. In the conventional manufacturing of soy sauce, a ratio of 1:3

soybean to water (w/v) was used (Hesseltine and Wang, 1979). In order to obtain a higher experimental accuracy for optimization purpose, an M/S proportion of, 1:10 was employed However, once optimum conditions for enzymatic hydrolysis had been established the higher ratio of 1:5 (w/v) was used in subsequent experiments of sauce production as it represented the point where the TSN curve entered the plateau (Figure 29).

According to the technical data supplied by the company, there were two ways to inhibit the enzyme activity. Firstly, the enzyme-substrate mash was brought to the temperature above 75°C for at least 30 min. Secondly, the mash was adjusted to a very acidic condition, about pH 3 - 4 for 10 min. Neither of these methods appeared to be appropriate for the present experiment, i.e., to obtain a maximal amount of TSN. High temperature employed in the former method would lead to the coagulation of soluble protein to insoluble form, whereas, the latter might hinder subsequent mold propagation during koji preparation. Nevertheless, since extraction of liquid from the mash was done right after the hydrolysis, followed immediately with the TSN analysis, it was decided that enzyme inhibition was not necessary for this experiment, and that results among the samples would be comparable as long as the experiments were performed consistently.

Central Composite Rotatable Design was employed in the RSM. The technique was not used to gain understanding of the physical mechanism of the system, although it might assist in gaining such knowledge. The experimental design is said to be rotatable since the variance of the predicted response y at some point X(s) is a function only of the distance of the point from the centre, and not a function of its direction. This implies that the variance contours of y are concentric circles. Furthermore, a design with this property will leave the variance of y unchanged when the design is rotated about the centre (0,0,0), hence the name rotatable design (Montgomery, 1984). Five replicates were made at the central coding condition (0,0,0), i.e., temperature of 68 C, pH 9 and enzyme-substrate ratio of 0.3 (v/w). This was used to test the lack of fit for the entire experiment.

Table 5 showed the yields of TSN with the corresponding values of temperature, pH and enzyme-substrate ratio.

The second order polynomial equation obtained from the analysis of multiple regression on the coded data (Table 6) was

$$\begin{aligned} y = {} & 0.479 + 0.0083X_1 + 0.00077X_2 + 0.011X_3 \\ & - 0.015X_1^2 - 0.020X_2^2 - 0.023X_3^2 + 0.012X_1X_2 + \\ & 0.0051X_1X_3 + 0.00073X_2X_3 \end{aligned}$$

Examination of the fitted model with student's t-test indicated that all quadratic terms ($X_1^2$, $X_2^2$, $X_3^2$)with one linear term ($X_3$) and one interaction term ($X_1X_3$) were significant at 95% level. The F-value and the correlation coefficient of the overall model was 11.27 and 0.96 which

Table 5. Observed and predicted results of percentage total soluble nitrogen

| Levels | | | % Total soluble nitrogen (% TSN) | |
|---|---|---|---|---|
| Temp. ( C) | PH | E/S (v/w) | Observed | Predicted |
| 63.8 | 8.4 | 0.24 | .4252 | .4354 |
| 72.2 | 8.4 | 0.24 | .4167 | .4130 |
| 63.8 | 9.6 | 0.24 | .4026 | .4107 |
| 72.2 | 9.6 | 0.24 | .4241 | .4347 |
| 63.8 | 8.4 | 0.36 | .4398 | .4318 |
| 72.2 | 8.4 | 0.36 | .4352 | .4297 |
| 63.8 | 9.6 | 0.36 | .3980 | .4042 |
| 72.2 | 9.6 | 0.36 | .4560 | .4484 |
| 61.0 | 9.0 | 0.30 | .4401 | .4315 |
| 75.0 | 9.0 | 0.30 | .4450 | .4499 |
| 68.0 | 8.0 | 0.30 | .4035 | .4089 |
| 68.0 | 10.0 | 0.30 | .4131 | .4040 |
| 68.0 | 9.0 | 0.20 | .4494 | .4357 |
| 68.0 | 9.0 | 0.40 | .4341 | .4442 |
| 68.0 | 9.0 | 0.30 | .4882 | .4797 |
| 68.0 | 9.0 | 0.30 | .4861 | .4797 |
| 68.0 | 9.0 | 0.30 | .4716 | .4797 |
| 68.0 | 9.0 | 0.30 | .4746 | .4797 |
| 68.0 | 9.0 | 0.30 | .4774 | .4797 |

Table 6. Results of %TSN with coded data

| Temp.<br>(X1) | PH<br>(X2) | E/S<br>(X3) | Temp.<br>($X1^2$) | PH<br>($X2^2$) | E/S<br>($X3^2$) | Temp. PH<br>(X1X2) | Temp. E/S<br>(X1X3) | PH E/S<br>(X2X3) | %TSN/g sample |
|---|---|---|---|---|---|---|---|---|---|
| -1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | .4252 |
| 1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | .4167 |
| -1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | .4026 |
| 1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | .4241 |
| -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | .4398 |
| 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | .4352 |
| -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | .3980 |
| 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | .4560 |
| -1.682 | 0 | 0 | 2.829 | 0 | 0 | 0 | 0 | 0 | .4401 |
| 1.682 | 0 | 0 | 2.829 | 0 | 0 | 0 | 0 | 0 | .4450 |
| 0 | -1.682 | 0 | 0 | 2.829 | 0 | 0 | 0 | 0 | .4035 |
| 0 | 1.682 | 0 | 0 | 2.829 | 0 | 0 | 0 | 0 | .4131 |
| 0 | 0 | -1.682 | 0 | 0 | 2.829 | 0 | 0 | 0 | .4494 |
| 0 | 0 | 1.682 | 0 | 0 | 2.829 | 0 | 0 | 0 | .4341 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | .4882 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | .4861 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | .4716 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | .4746 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | .4774 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |

were also significant at 95% level. The lack of fit of the experiment was insignificant. All these implied that the fitted model was appropriate for the description of the response surface. With the regression coefficient obtained, stationary point of the fitted surface was computed according to the equations suggested by Smith (1982). The predicted yield y together with the coded and natural variables of the stationary point ($X_0$), which were well within the experiment limits, are shown in Table 7.

To characterize the stationary point, canonical analysis was performed on the second order polynomial equation. The analysis was used to transform the fitted model to a new co-ordinate system with the origin at the stationary point ($X_0$), and then the axes of the system were rotated until they were parallel to the principal axes of the response surface. The transformation was performed by means of the computer program in APL language.

The canonical form of the second order polynomial equation demonstrating the nature of the response surface was given by

$$y = 0.4813 - 0.01604W_1^2 - 0.02188W_2^2 - 0.02528W_3^2$$

where $W_1$, $W_2$, $W_3$ were the axes of the response surface. Note that all the eigenvalues (-0.01104, -0.02188, -0.02525) were negative indicating that the stationary point was in fact a

Table 7. The coded and uncoded variables of the stationary point

| Variables | | Coded | uncoded |
|---|---|---|---|
| Temperature | (X1) | 0.232 | 69.0 |
| PH | (X2) | 0.216 | PH 9 |
| E/S | (X3) | 0.131 | 0.31 (v/w) |

predicted yield = .4813 %TSN

observed yield = .4882 %TSN

maximum with the surface slightly extended to the W1 axis (Myers, 1971). Three dimensional graph fixing one variable at a time were plotted, as shown in Figures 32 to 34.

Using the condition obtained at the stationary point (69 C, pH 9 and enzyme-substrate ratio of 0.31), the experimental result of %TSN was found to be 0.4882 which closely agreed with the computed optimal yield of 0.4813.

### 4.2 Koji and Moromi Preparations

Results obtained from the previous experiment (Table 7) showed that the optium conditions for canola meal hydrolysis by Alcalase 0.6 L were : 69°C, pH 9, E/S ratio of 0.31 (v/w), and 2 h reaction time. Meal-solvent ratio of 1:5 instead of 1:10 (v/w) was employed in the enzymatic hydrolysis due to the reasons stated earlier.

Sodium hydroxide, automatically fed in by the pH-stat, instead of buffer solution, was used in this experiment so that ionic strength of the mash would be lower and higher TSN could be produced. A yield of 0.848% TSN instead of 0.488%TSN as shown in the optimization experiment was obtained when the enzyme hydrolyzate was prepared with NaOH. The substantially high result of %TSN might also be attributed to the low M/S ratio employed.

In order to have an appropriate mold propagation, pH of the mash was adjusted to 5.5 and the liquor was extracted so that around 40 -45% moisture would be retained in the mash. It was noted that occasional stirring of the mash during

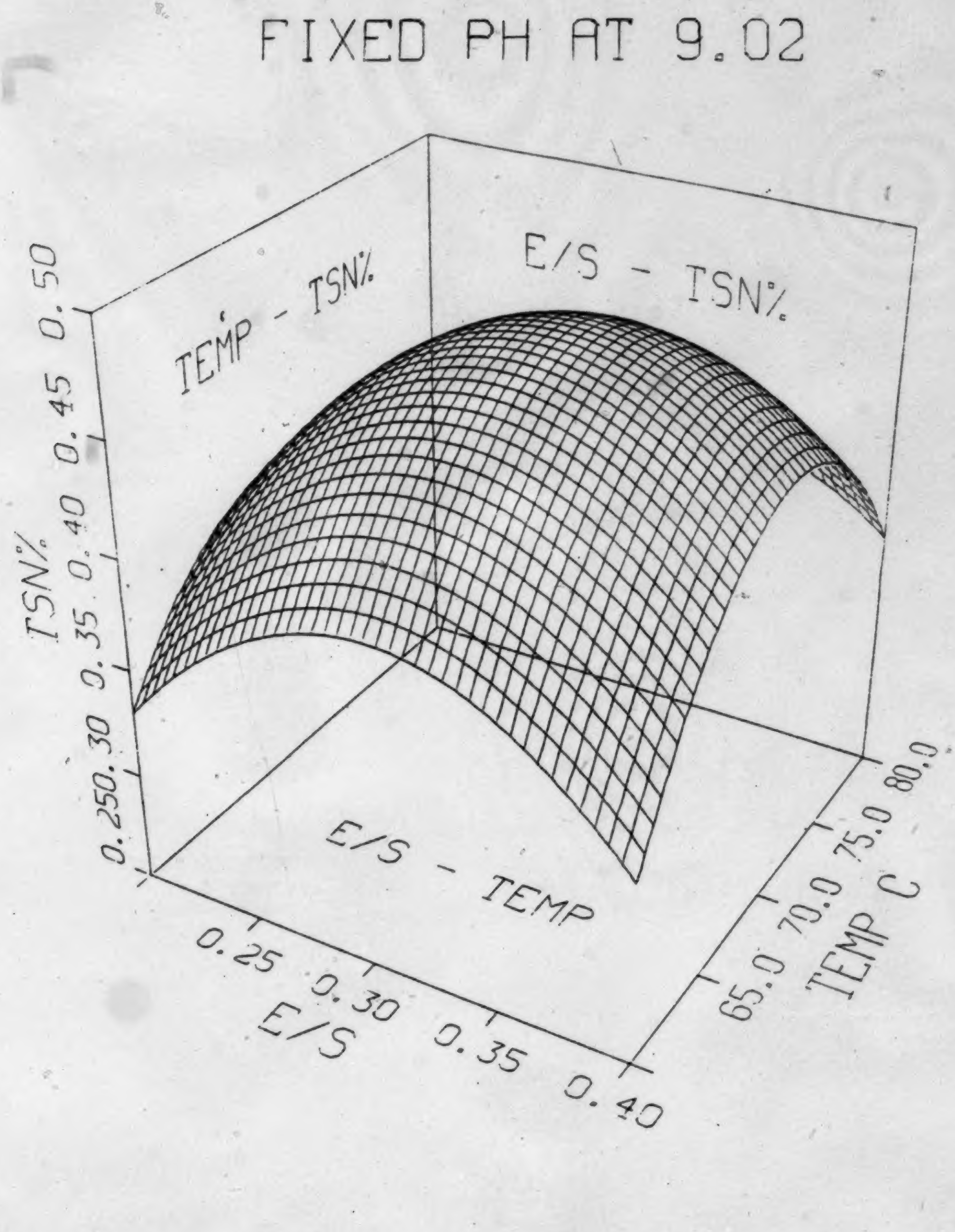


Figure 32 Three dimensional illustration on optimal conditon for enzymatic hydrolysis on canola meal. PH was fixed at 9.02

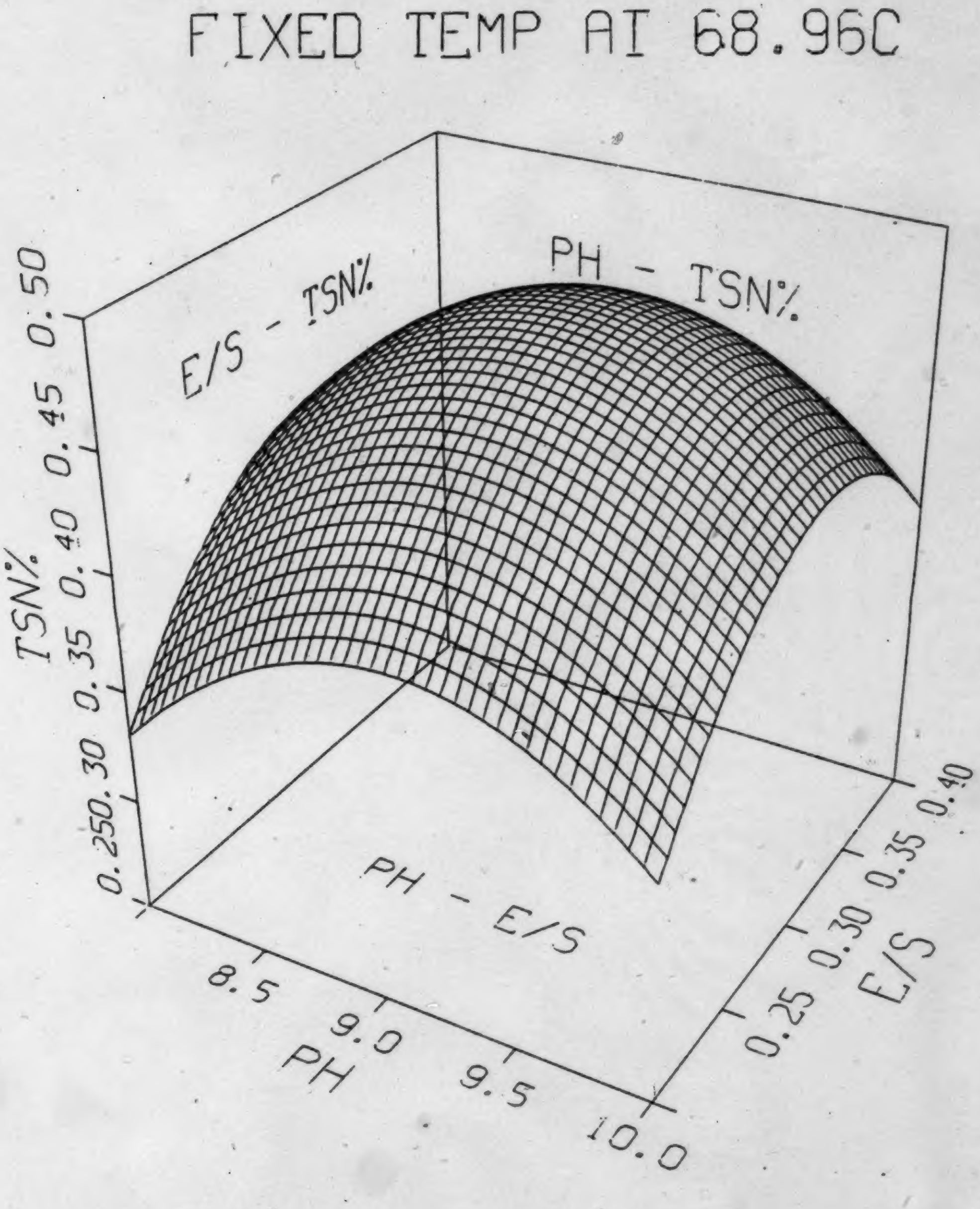


Figure 33 Three dimensional illustration on optimal conditon for enzymatic hydrolysis on canola meal. Temperature was fixed at 68.96 C.

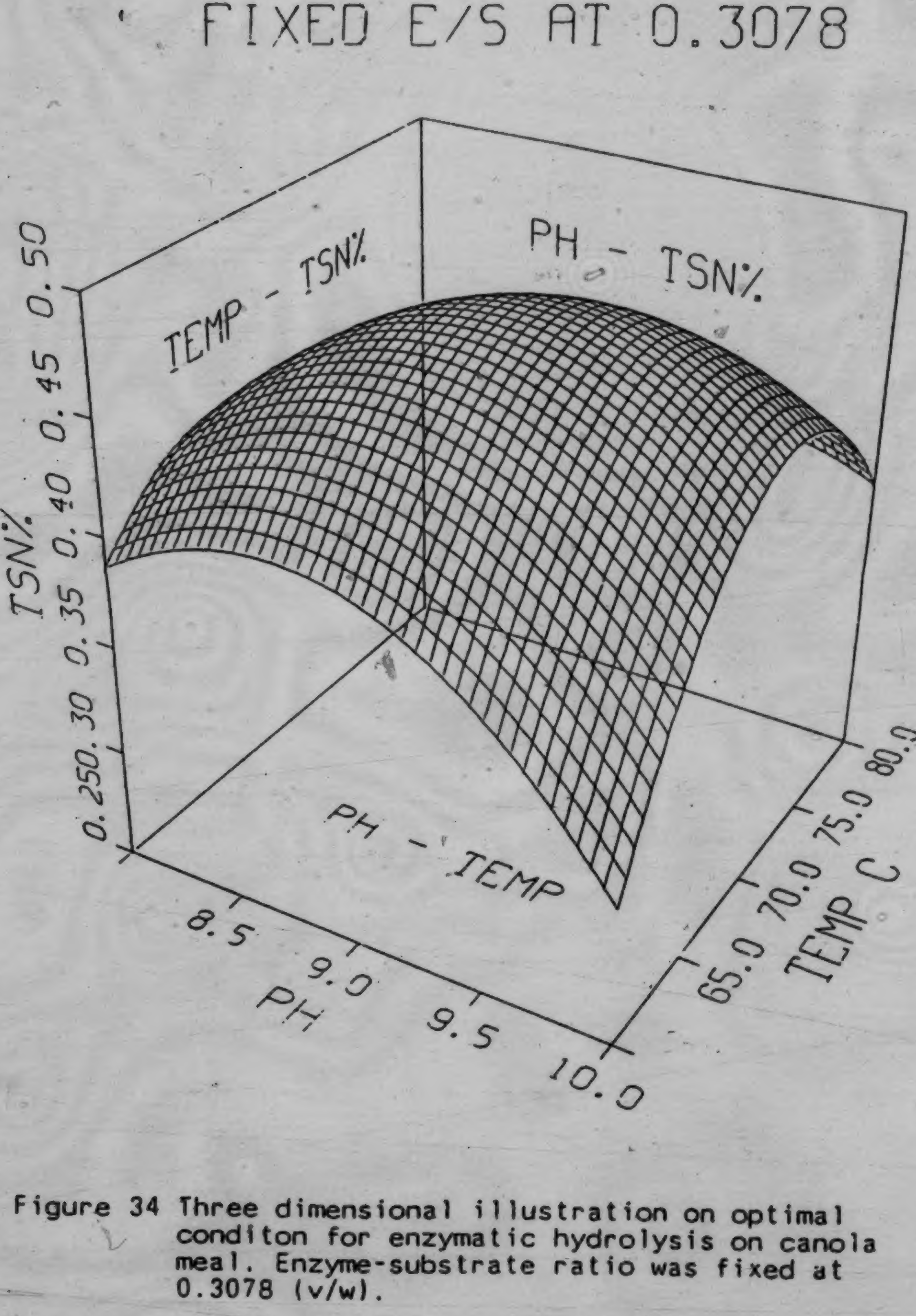


Figure 34 Three dimensional illustration on optimal conditon for enzymatic hydrolysis on canola meal. Enzyme-substrate ratio was fixed at 0.3078 (v/w).

fermentation was necessary in order to aerate it for proper mold growth.

To prepare the koji, mash residue was mixed with roasted ground wheat and *Aspergillus* culture and incubated at 28°C for 72 h.. Four samples inoculated with different cultures were investigated. These included 2 two sample inoculated with *A.oryzae*, one with *A.sojae* and one with a combination of *A.oryzae* and *A.sojae* mash.

After incubation, the koji was covered with greenish yellow mycelia with a sweetish and a slight ammonia smell. Its moisture content dropped from 40 - 45% to around 28%.

Since all the required solvent had already been added to the canola meal during the enzymatic hydrolysis, further addition of brine to prepare moromi would result in excessive dilution of the sauce. Salt was, therefore, added to the extracted liquor (enzyme hydrolyzate) in order to prepare the 18% brine. The salted enzyme hydrolyzate was then added back to the mash to facilitate subsequent fermentation. It would served as an immediate source of nutrient for the bacterial growth. Soluble peptides were further broken down to amino acids and other nitrogenous compounds while simple sugars were converted into organic acids during the fermentation. One of the *A.oryzae* inoculated kojis was adjusted to pH 6.5 in order to study the effect of the acid fermentation. The remaining three koji samples were adjusted to pH 5.5. The pH was expected to drop to below pH 5 after the acid fermentation so that yeast growth could be initiated.

### 4.3 Changes in total soluble solids and pH during Moromi Fermentation

Fermentation of moromi in this experiment relies on the spontaneous growth of natural lactic acid bacteria and, perhaps, yeast. The total soluble solid (TSS) contents of most of the moromi liquors increased steadily from 28% to 35% during the 5 weeks incubation except the one inoculated with *A.oryzae* with pH adjusted to 5.5, where the TSS content began at 32% and then rose to 38% after 5 weeks. All samples had a relatively rapid increase in soluble solids within the first one to two weeks of fermentation after which the increase became more gradual until the plateau was reached in the fourth week(Figure 35).

Ooraikul et al. (1980) reported that there were two rises of TSS in canola sauce produced by the the conventional fermentation. The first rise was interpreted as due to the acid fermentation by bacteria while the second rise was accounted for by alcoholic fermentation of the yeast. It is doubtful that the two rises of the TSS in the moromi with *A.oryzae* and initial pH of 6.5 shown in the graph (Figure 35) were the same as those reported by Ooraikul et al. (1980). Yeast fermentation could not have been initiated since the pH of the mash was still higher than 5.0 as was the case in all five samples tested (Yong and Wood, 1977b). The two rises observed, therefore, might

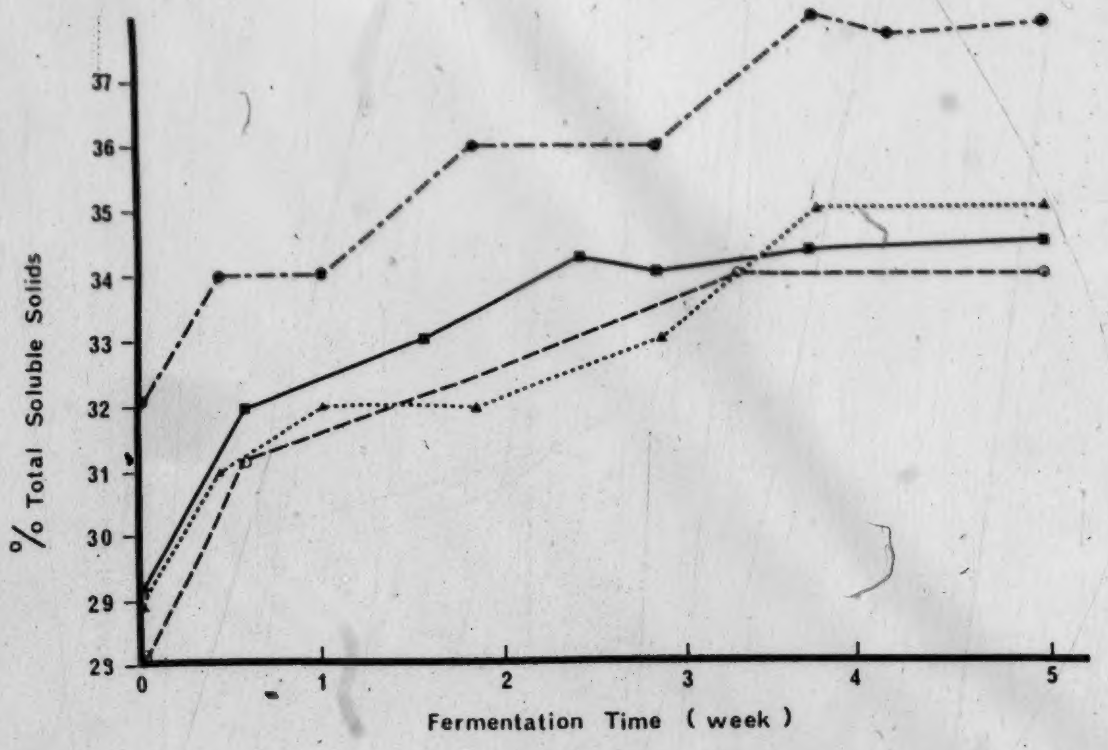


Figure 35 Changes in total soluble solids during enzymatic production of canola sauce
( ● )CS1, canola sauce prepared with A. oryzae at pH 5.5
( ■ )CS2, canola sauce prepared with A. sojae at pH 5.5
( o )CS3, canola sauce prepared with A. oryzae & A. sojae at pH 5.5
( ▲ )CS4, canola sauce prepared with A. oryzae at pH 5.5

be due to the fluctuation of growth of other microorganisms during the fermentation.

Sarkar et al. (1979) reported that the increase in soluble solids during the first ten weeks of conventional fermentation was due mainly to acid fermentation where carbohydrates in the substrates were converted to sugars and organic acids and proteins to amino acids. The production of acids would account for the gradual decrease in pH. The results in this experiment support their findings since the increase in TSS in the first one to two weeks of fermentation was accompanied by a gradual decline of pH in the mashes (Figure 36). There was only slight decrease of pH from 5.5 to 5.2 in the sample with *A.oryzae* and the one inoculated with a combination of *A. oryzae* and *A.sojae*. The *A.sojae* inoculated mash, on the other hand, did not show any detectable drop in pH. A more noticeable decline was found in the mash prepared with *A.oryzae* with the initial pH of 6.5 where a drop from 6.5 to 5.6 was observed in the first two weeks of incubation. In all samples, pH approached its minimum after the second week and remained basically unchanged until the fermentation was terminated (Figure 36). The very slow decrease in pH in the mashes might be attributed to the high buffering capacity of the moromi and/or the deficiency of lactic acid fermentation. The acid fermentation would have been more prominent if lactic acid bacteria were inoculated into the mash.

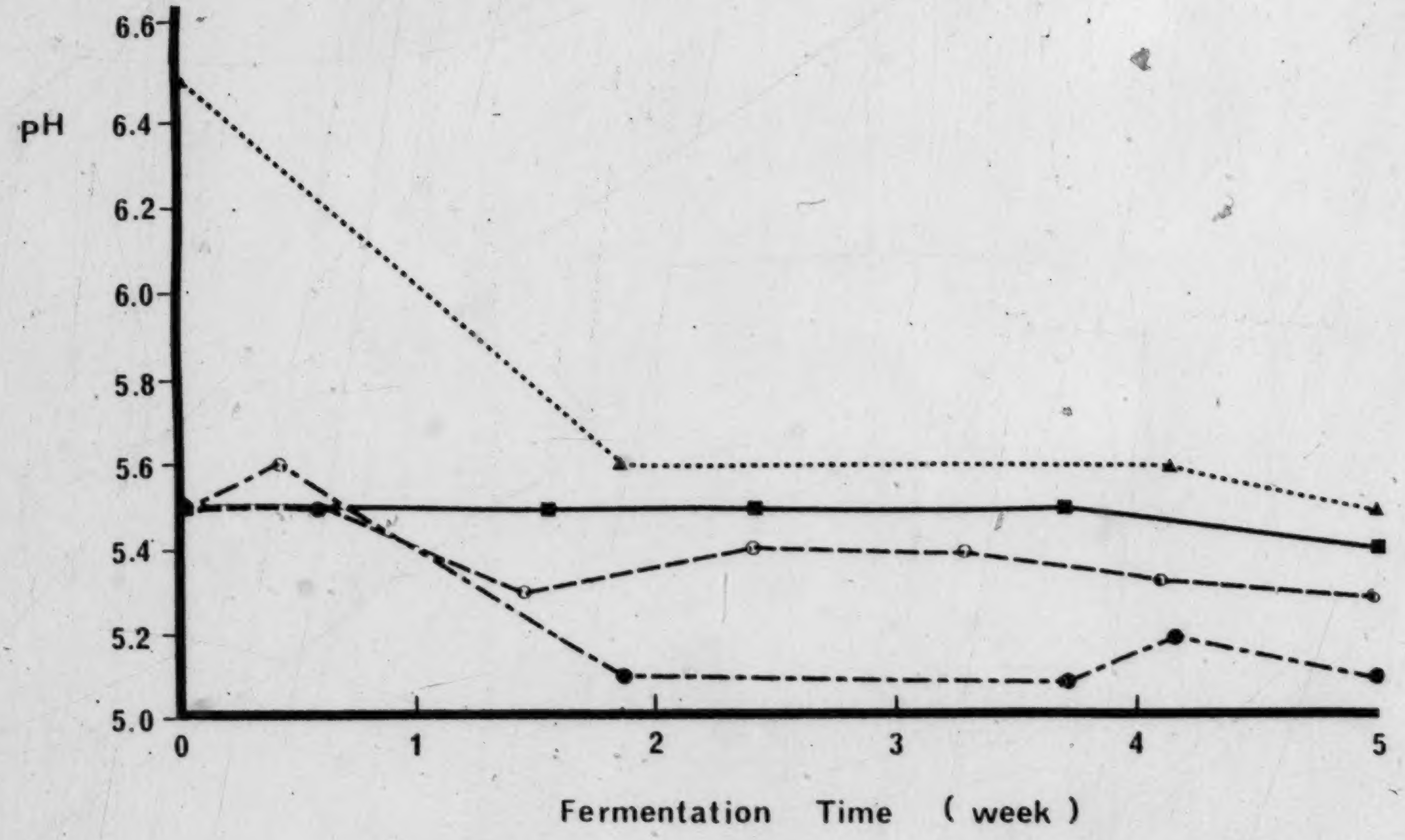


Figure 36 Changes in pH during enzymatic production of canola sauce

When the results are compared with the TSS and pH of the mash prepared by partial acid hydrolysis (42% and 5.2, respectively) as reported by Ooraikul et al., (1980), the final TSS contents of the sample in this experiment seemed to be lower while the pH was comparable. However, it should be noted that 1:3 meal-solvent ratio was used in the preparation of acid hydrolyzate while 1:5 was used in the enzymatic hydrolysis. If soluble solids concentration of the two mashes were calculated based on the same solvent - meal ratio, they would be quite similar or, in fact, slightly higher in the enzyme-prepared moromi. higher.

Comparable %TSS (36% vs. 34-38%) were found between a commercial soy sauce and the samples produced in this experiment. However, pH of the commercial product was 4.9 as compared to 5.1 - 5.6 of the canola sauce samples. It is quite obvious that greater degree of acid fermentation occurred in the commercial product in which conventional fermentation method with an 'artificial' inoculation of lactic acid bacteria was employed.

### 4.4 Composition of Canola Sauces

#### 4.4.1 Nitrogen Containing Compounds

4.4.1.1 Total soluble nitrogen (TSN) and amino nitrogen (AN)

Table 8 shows the TSN and AN contents of the canola sauce, commercial soy sauce as well as the enzyme hydrolyzate. Nitrogen yield and AN/TSN ratios are also

Table 8. Results of nitrogen containing compounds

| Samples[a] | %TSN[b] (w/w) | %nitrogen[e] yield | %AN[c] (w/v) | %AN/TSN |
|---|---|---|---|---|
| CS1 | 1.31 | 71.74 | 0.80 | 61.06 |
| CS2 | 1.16 | 63.53 | 0.70 | 60.34 |
| CS3 | 1.34 | 73.38 | 0.66 | 48.50 |
| CS4 | 1.12 | 61.34 | 0.73 | 65.18 |
| K | 1.38 | 73.70[d] | 0.70 | 58.72 |
| EH | 0.85 | 46.54 | 0.64 | 75.29 |

[a] CS1 = canola sauce prepared with A.oryzae at pH 5.5
CS2 = canola sauce prepared with A.sojae at pH 5.5
CS3 = canola sauce prepared with A.oryzae & A.sojae at pH 5.5
CS4 = canola sauce propared with A.oryzae at pH 6.5
K = commercial soy sauce produced by Kikkoman shoyu Co. Ltd.
EH = Alcalase 0.6L - canola meal enzyme hydrolyzate

[b] %TSN = percentage total soluble nitrogen
[c] %AN = percentage amino nitrogen

[d] Obtained from Smith and Circle, 1978.

[e] %nitrogen yield = TSN in sauce / nitrogen content in raw materials
nitrogen content per 100g raw materials = 4.565g

presented. These two characteristics serve as quality as well as price indices for commercial soy sauce.

TSN contents of the canola sauce samples varied from 1.12 to 1.34% which were slightly lower than that of a commercial soy sauce sample (Table 8). A concentration of 1.5% TSN in Koikuchi shoyu, which is slightly higher than that of the commercial sample used in this experiment, was reported by Yokotsuka, (1981). However, %TSN in soy sauce varied from brand to brand, and sometimes even from year to year of the same brand. The TSN levels of the canola sauce prepared in this experiment were, therefore, considered comparable to that of the commercial products.

Samples inoculated with *A. oryzae* at pH 5.5 seemed to have relatively high %TSN contents, whereas that prepared with inoculum but at pH 6.5 gave the lowest %TSN. This indicated the important effect of pH on moromi fermentation.

High nitrogen yield denotes an effective enzymatic conversion of raw material protein to soluble form. Smith and Circle (1978) reported that nitrogen yield on commercial soy sauce was 73.% , which was used as the nitrogen yield in Kikkoman shoyu. Samples inoculated with *A.oryzae* (CS1) and a combination of *A.oryzae* and *A.sojae* (CS3) at pH 5.5 appeared to give nitrogen yields comparable to that of the commercial product.

Degradation of proteins to soluble nitrogen in this experiment could be roughly divided into two stages: (1) enzymatic prehydrolysis of canola meal done by Alcalase 0.6L and (2) further degradation of proteins and peptides by extracellular protease liberated from Aspergillus molds. More than half of the nitrogen yield in the canola sauce was attributed to the enzymatic action. Therefore, enzymatic prehydrolysis of raw materials appeared to be an effective means to ensure high soluble nitrogen content in the sauce product.

Canola sauce prepared in this experiment contained amino nitrogen levels ranging from 0.66% to 0.80%, which agreed well with the literature value of 0.88% in the commercial Koikuchi shoyu (Fukushima, 1979). Again, as in the case of %TSN, high %AN was obtained in enzyme hydrolyzate (Table 8) indicating the importance of the enzyme in canola meal prehydrolysis.

Almost all of the samples had very high AN/TSN ratios. A ratio of greater than 50% amino nitrogen to tota soluble nitrogen is regarded as an evidence of good quality soy sauce. Therefore, in terms of TSN, nitrogen yield, AN and AN/TSN ratio, canola sauce prepared in this experiment appeared to have quality comparable to that of commercial soy sauce. Among the various samples prepared, the one fermented with *A.oryzae* at pH 5.5 appeared to give results most closely resembling those of the commercial product.

#### 4.4.1.2 Amino acid profile

Amino acids and their concentration in various sauce samples are shown in Table 9. Among the 16 amino acids analyzed, glutamic acid and aspartic acid were the two major

Table 9. Contents of amino acids in canola sauces and soy sacue.

| | CS1 | CS2 | CS3 | CS4 | K | EH |
|---|---|---|---|---|---|---|
| Asp. A. | 24.40 | 34.50 | 35.50 | 39.50 | 65.70 | 4.35 |
| Thr. | 14.40 | 16.70 | 24.25 | 19.85 | 30.60 | 1.95 |
| Ser. | 16.45 | 29.45 | 31.40 | 31.50 | 52.35 | 4.30 |
| Glu. A. | 61.85 | 83.40 | 87.75 | 76.80 | 86.55 | 9.65 |
| Pro. | 20.80 | 25.25 | 34.25 | 21.85 | 52.00 | 6.35 |
| Gly. | 20.35 | 25.10 | 28.90 | 28.90 | 37.05 | 3.40 |
| Ala. | 37.05 | 40.00 | 47.75 | 43.95 | 55.65 | 6.75 |
| Cys. | 6.20 | 7.35 | 8.75 | 7.00 | 3.40 | 4.90 |
| Val. | 29.80 | 38.35 | 43.50 | 38.80 | 55.95 | 6.70 |
| Met. | 13.60 | 12.10 | 17.15 | 11.90 | 10.05 | 5.85 |
| Ile. | 19.05 | 23.55 | 26.55 | 23.90 | 41.30 | 4.70 |
| Leu. | 34.45 | 41.75 | 29.10 | 40.15 | 65.10 | 4.70 |
| Tyr. | 11.20 | 13.75 | 16.15 | 13.15 | 6.90 | 8.15 |
| Phe. | 16.45 | 18.65 | 21.55 | 17.00 | 31.05 | 1.70 |
| Lys. | 19.35 | 23.85 | 29.10 | 24.05 | 49.05 | 3.95 |
| Arg. | 17.65 | 25.70 | 30.55 | 25.75 | 21.75 | 2.00 |

Concentrations of amino acids were expressed in mMoles/mL sauce amino acids in the products. According to Udo (1931), these two acids were responsible for the delicious taste in soy sauce. From the chromatograms, the peak of ammonia was so big that it overlapped with the histidine peak. Therefore, histidine content could not be measured in this analysis. In general, the contents of amino acid in the commercial product were higher than those of the canola sauce. Cystine, tyrosine and methionine were the only three amino acids in the canola sauce higher than those of the soy sauce. However, the concentration of glutamic acid, probably the most important amino acid that contributes to the charactistic taste, was quite similar in both canola and soy sauce.

A substantial increase in free amino acids occurred during koji incubation as well as moromi fermentation. This was well illustrated by the difference in amino acid levels of the enzyme hydrolyzate and the sauce obtained after the fermentation. Together with the results of nitrogen yields in both enzyme hydrolyzate and fermented sauce samples, it could be suggested that the sequence of protein hydrolysis in canola sauce processing was as follows: complex proteins in the raw materials were broken into small peptides during the enzymatic prehydrolysis, followed by further proteolytic breakdown of the small peptides and remaining proteins into free amino acids and other soluble nitrogen by mold proteases during koji and moromi stages.

### 4.4.2 Titratable Acidity and Organic Acid Profile

Relativley low total titratable acidity was obtained in all the canola sauce samples, as compared with that of the soy sauce sample (Table 10). These results were closely reflected in the slow decline of pH in moromis during fermentation. As mentioned earlier, deficient acid fermetnation due to the absence of artificial inoculation of lactic acid bacteria might account for the low acidity in the samples. Total acid existed in the sauce fermented with *A.sojae* (16.65 meq.NaOH/100mL) was assumed to be essentially the result of the enzymatic prehydrolysis since there was no noticeable increase in acidity after the hydrolysis (i.e. that of EH = 16.30 meq. NaOH/100mL) nor was there any significant change in pH during fermentation (Figure 36).

Onaga et al. (1957) analyzed the chemical composition of 7 brands of soy sauce obtained from the market. Total acidity of the 7 samples varied from 6.13 to 19.97 meq NaOH/100mL sauce, which were lower than that of the results obtained in this experiment. The discrepancy might be due to the fact that straight titration was performed without any fractionation treatment by Onaga et al (1957). Alkali substances present in the samples might interfere with the titration and therefore lower total acidity obtained.

Examination of the HPLC chromatogram on organic acids of the sauce samples revealed that 11 major peaks were found in the profiles. Among them, peaks number 9, 10 and 11 were the most prominant peaks and were identified to be a mixture of lactic, formic acid and acetic acids, propionic acid, respectively. Each individual peak (Figure 37-41) was identified by spiking the sample with standard organic acids. The acids present in the sauce were citric acid, isocitric acid, pyruvic acid, malic acid, trans-aconitic, glyoxylic, succinic, fomic, lactic, propionic acid and two unidentified organic acids. Peak number 9 was suspected to consist of a mixture of lactic and acetic acids. The two acids had very similar retention time and the lactic acid peak was so big that it might totally overshadow the smaller acetic acid peak. It has been reported by Kihara (1938) that the concentration of lactic acid present in soy sauce far outweighed that of acetic acid. Therefore, it is reasonable to regard lactic acid as the major acid in peak number 9. The distribution pattern of organic acids in the profiles of all the sample were remarkably similar to one another.

Organic acid bouquet of soy sauce was attributed to lactic, acetic, succinic and phosphoric acids (Yokotsuka, 1981). Among the 11 organic acids, lactic and formic were the two prominant acids in canola sauce (Table 10). Lactic acid was characteristically the most important in fermented soy sauce, whereas, levulinic and formic acids were the distinctive traits of chemical soy sauce (Fukushima, 1981). The canola sauce prepared with enzymatic approach had very high concentration of lactic and formic acids, while no levulinic acid was detected. The organic acid quality of

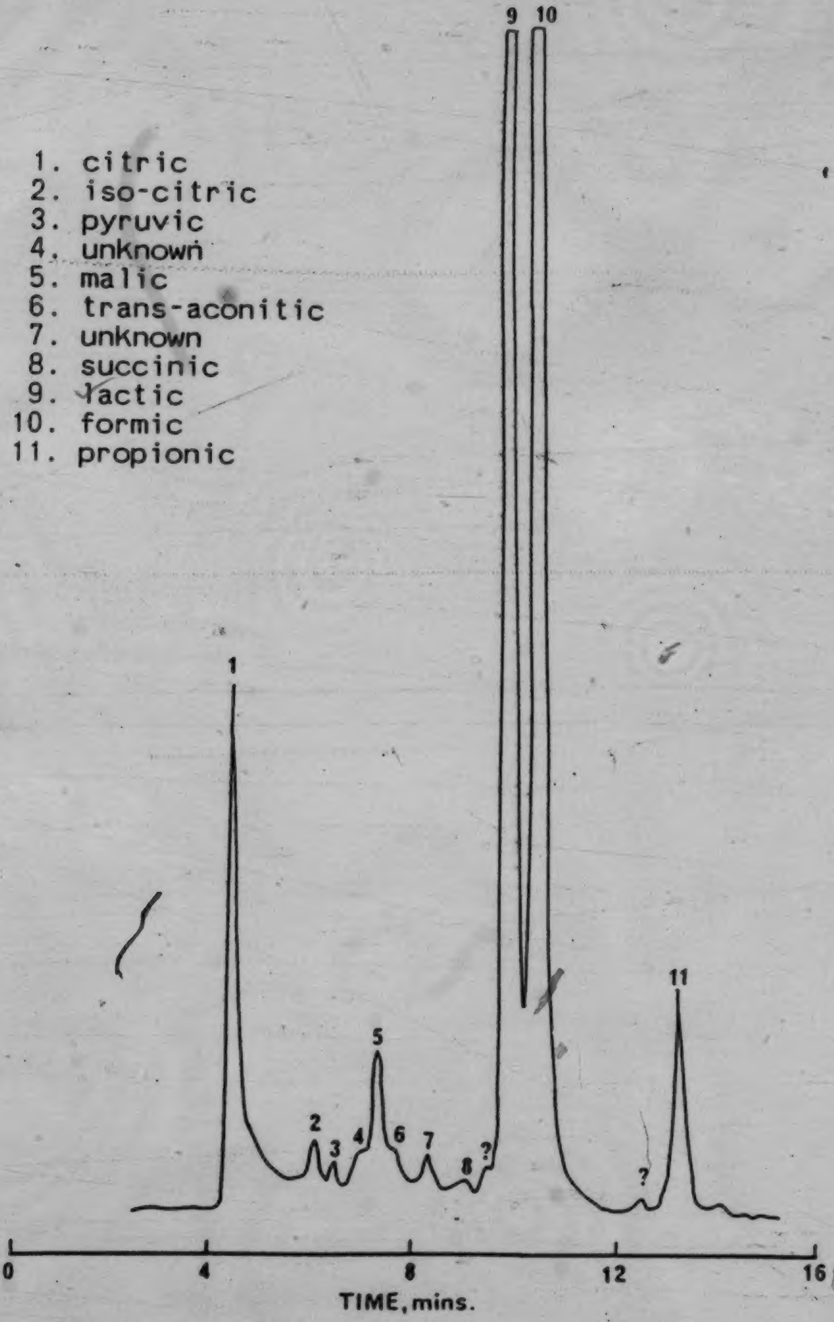


Figure 37. HPLC chromatogram of organic acid profile in canola sauce prepared with A. oryzae at pH 5.5

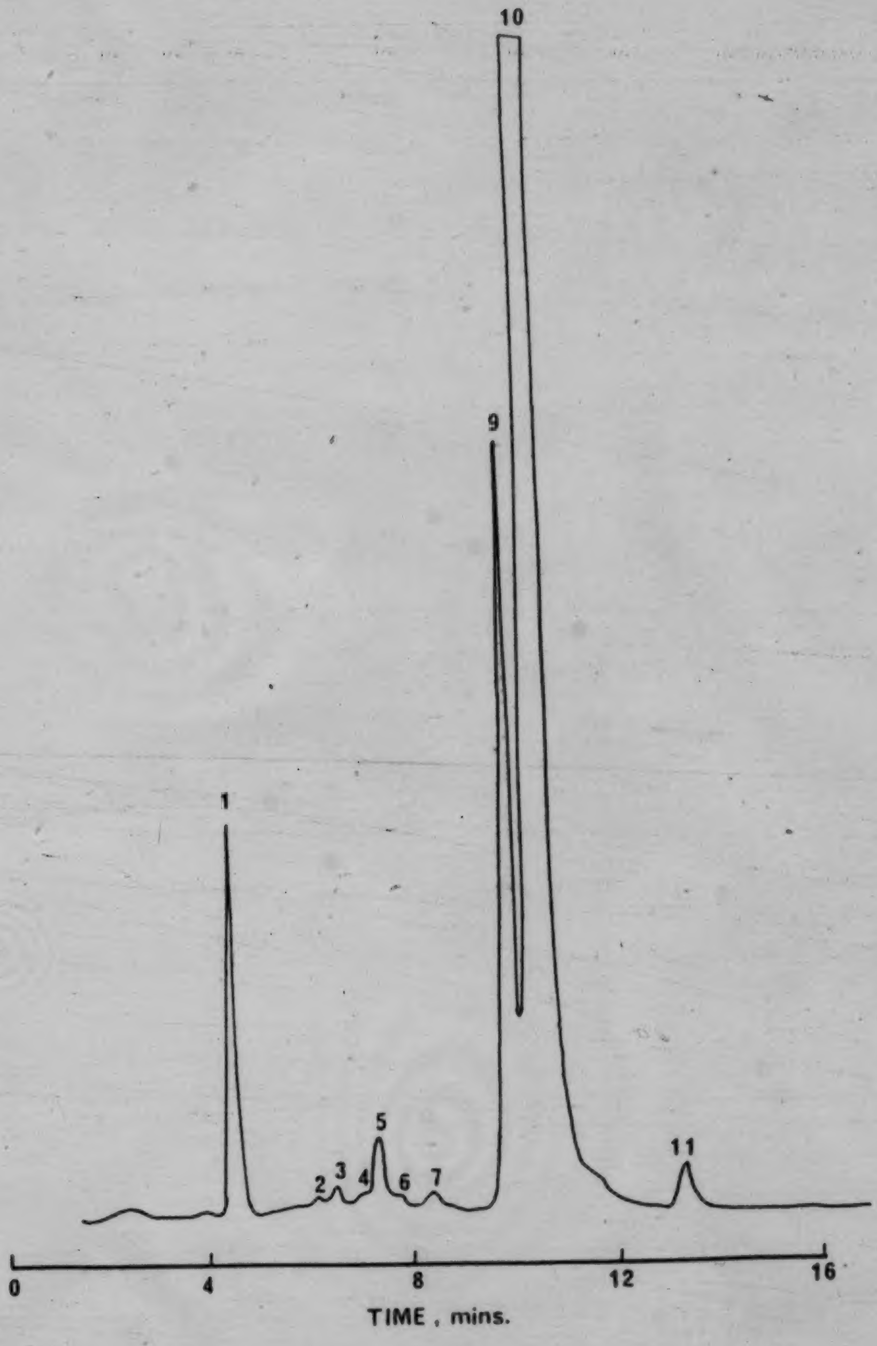


Figure 38. HPLC chromatogram of organic acid profile
in canola sauce prepared with A. sojae
at pH 5.5

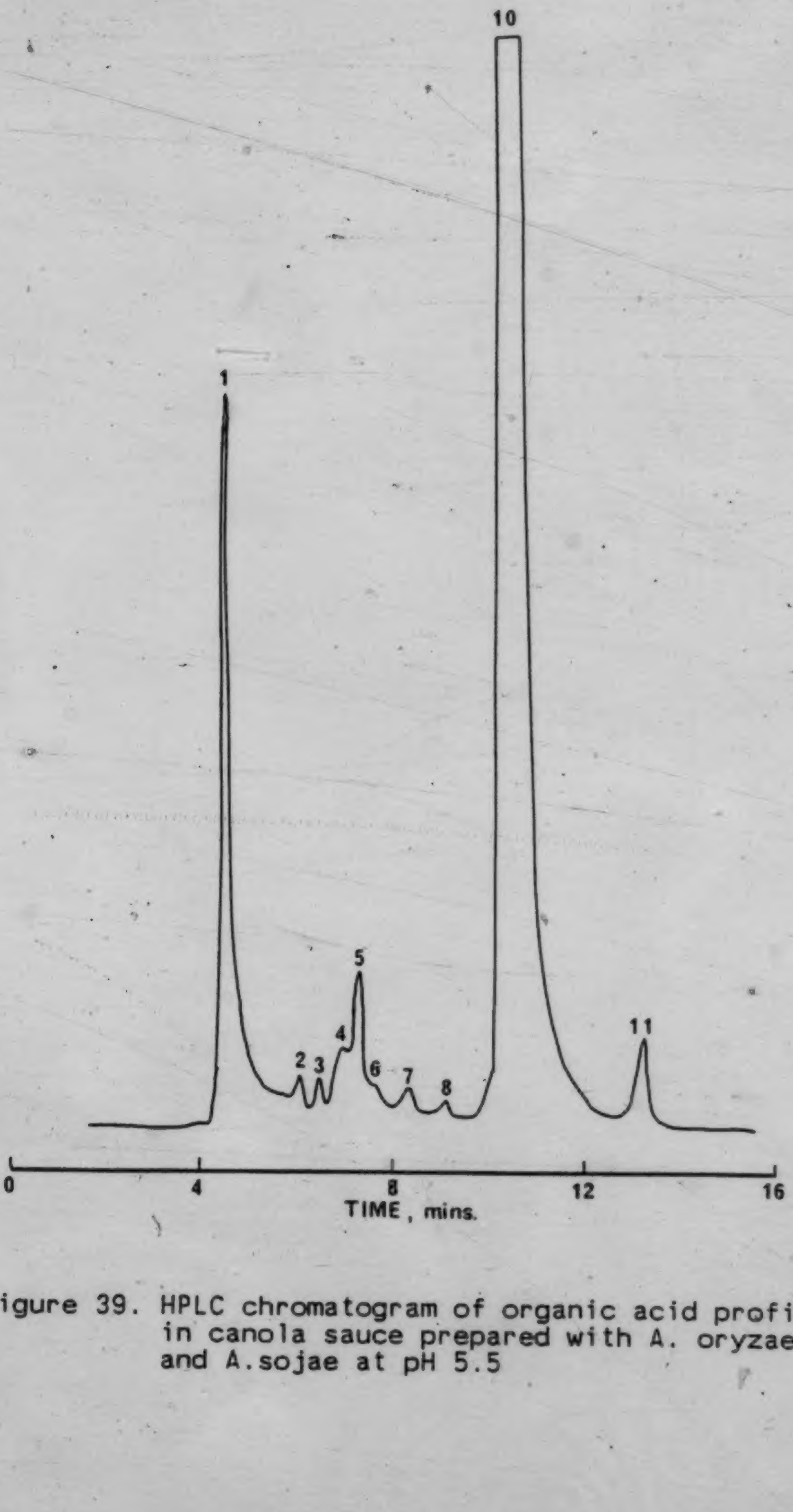


Figure 39. HPLC chromatogram of organic acid profile in canola sauce prepared with A. oryzae and A.sojae at pH 5.5

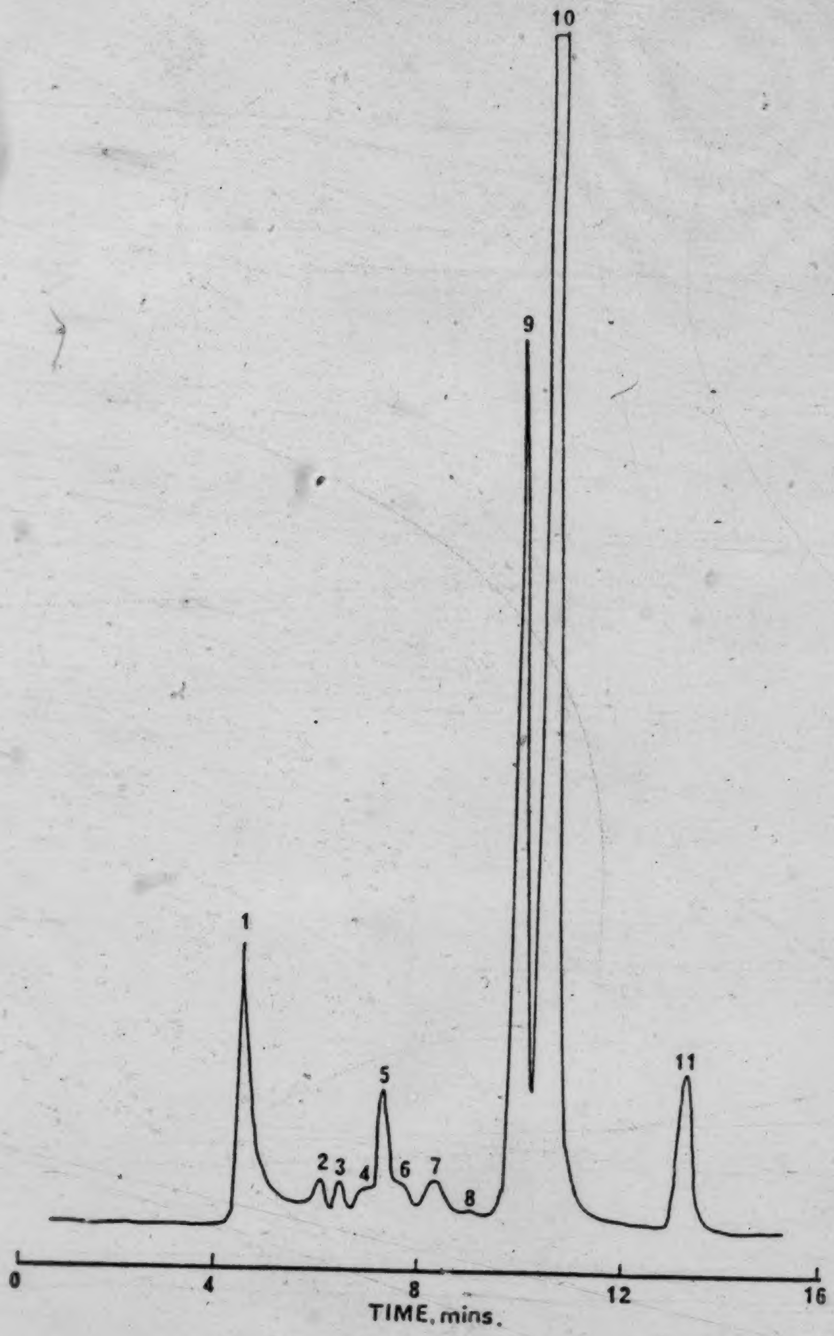


Figure 40. HPLC chromatogram of organic acid profile in canola sauce prepared with A. oryzae at pH 6.5

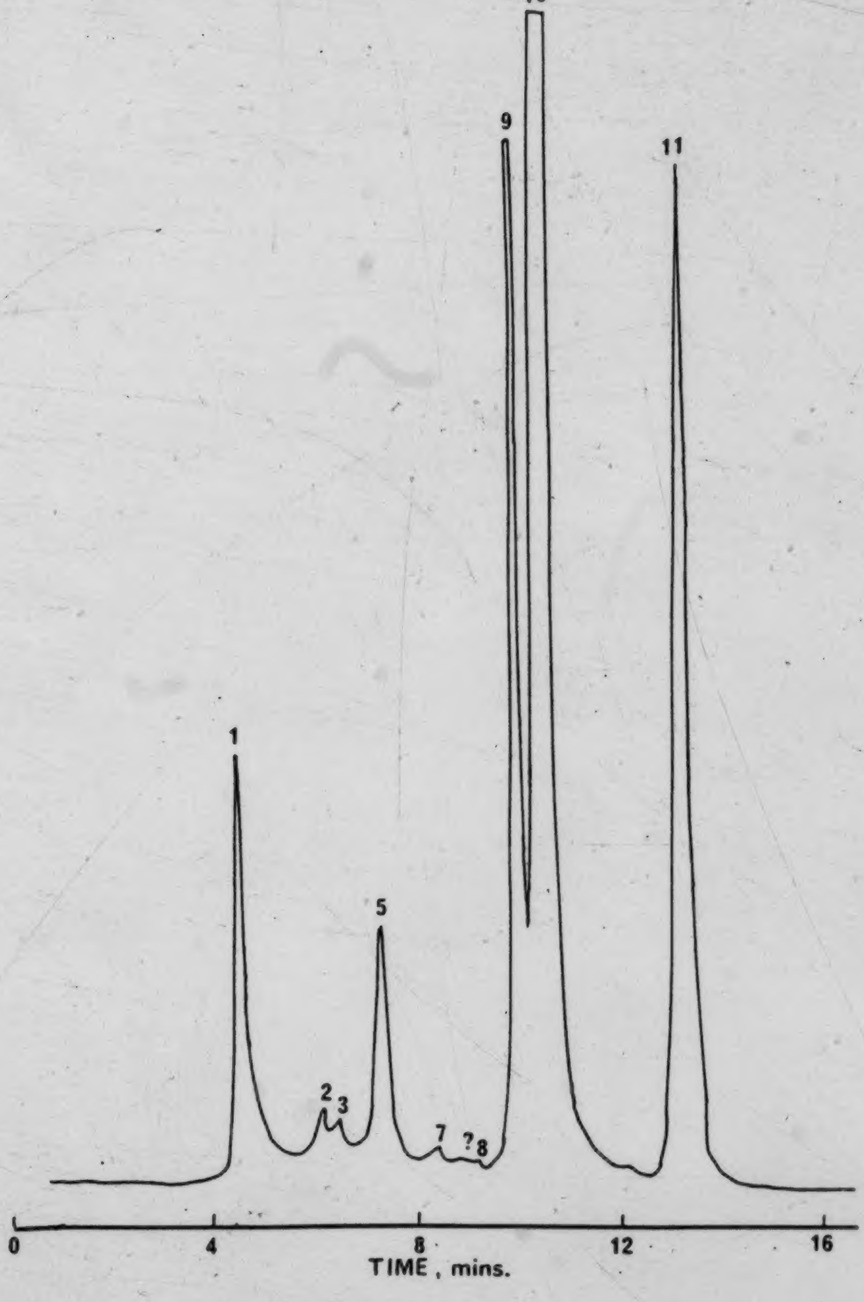


Figure 4.1 HPLC chromatogram of organic aicd profile in commercial soy sauce

Table 10. Total acidity and organic acid composition and concentration of canola sauce and soy sauce

| | CS1 | CS2 | CS3 | CS4 | K | EH |
|---|---|---|---|---|---|---|
| Total acidity meq.NaOH/100mL sauce | 24.40 | 16.65 | 23.45 | 29.95 | 33.35 | 16.30 |
| Citric acid | 8.60 | 6.50 | 12.46 | 4.6 | 7.13 | 1.48 |
| Isocitric acid | 2.55 | 0.55 | 2.18 | 1.71 | 3.03 | - |
| Pyruvic acid | 0.14 | 0.07 | 0.15 | 0.13 | 0.16 | - |
| Unknown 1 | ++ | - | ++ | + | - | - |
| Malic acid | 2.96 | 1.44 | 3.07 | 2.51 | 4.75 | - |
| Trans-aconitic acid | 0.03 | + | 0.02 | 0.02 | - | - |
| Unknown 2 | ++ | + | ++ | ++ | ++ | - |
| Glyoxlic acid | 0.08 | - | - | - | - | - |
| Succinic acid | 0.74 | - | 0.74 | 0.52 | + | |
| Formic acid | 14.12 | 16.76 | U/E | 6.86 | 16.87 | 0.57 |
| Lactic acid | 36.01 | 63.76 | U/E | 74.58 | 87.85 | - |
| Propionic acid | 10.90 | 2.20 | 3.8 | 7.6 | 484.9 | - |

a Organic acids were expressed in mg/100mL

. ++ significant amount

. + presence

. - absence

.U/E unable to estimate due to overlap

Sample abbreviations are the same as in Table 8.

quality of the canola sauce therefore appeared to be between those of the fermented and chemical soy sauce.

Examination of the organic acid composition indicated that most of the acids had concentration similar to that of the commercial soy sauce, except propionic and lactic acids which were present in lower quantities in canola sauce. The deficiency of lactic acid present in canola sauce again supported the conclusion that there was an inadequacy of lactic acid fermentation in the process. Formic acid, the second major organic acid present in canola sauce, had a concentration comparable to that of the fermented soy sauce. Absence of levulinic acid together with the relatively low quantity of formic acid in canola sauce might be responsible for its absence of the undesirable taste usually found in chemical soy sauce (Reddy et al. 1982).

It was not suprising that barely any organic acids were detected in the enzyme hydrolyzate since the commercial enzyme used in the prehydrolysis stage was mainly proteolytic enzyme. Again, it was an additional evidence to support the suggestion that most of the organic acids were produced during the moromi fermentation while amino nitrogen or amino acids were rapidly generated through the enzymatic prehydrolysis as well as the proteolytic breakdown of peptides achieved by the mold.

There is a slight difference between the concentrations of organic acids reported herein and the data in the literature (Kihara, 1938; Ueda et al., 1979). For example, succinic acid, pyruvic acid, lactic acid in the commercial soy sauce used in this investigation were lower than those in literature values. This could be ascribed to the different products and/or different analytical methods used.

### 4.4.3 Salt Concentration

Traditional soy sauce contains salt concentration of around 17 to 21%. Too high a salt concentration reduces with protein digestibility and soluble nitrogen yield, whereas, too low a concentration may lead to the risk of microbial contamination. Samples prepared in this project contained 18 to 20% salt. Table 11 shows the concentration of NaCl in various sauce samples.

### 4.4.4 Total and Reducing Sugar Contents

Reducing sugar concentration of the sauce was expressed in terms of glucose equivalent, whereas, that of non-reducing sugar was reported as sucrose equivalent. Total sugar content was therefore a combination of reducing and non-reducing sugar values. Literature data reported about 4 to 6% reducing sugar content in most of the fermented soy sauce. For example, the sauce produced by Noda Soy Sauce Company contain approximately 5.99% reducing sugar, while the koikuchi shoyu produced by Kikkoman Shoyu Company consisted of 3.8% reducing sugar. Canola sauce prepared by enzymatic prehydrolysis in this research contained 7 - 9% reducing sugar, while the commercial soy sauce sample was

Table 11. Salt contents of the canola sauces and the commercial soy sauce

| | Reading ( mv ) | %NaCl ( w/v ) |
|---|---|---|
| CS1 | 77.83 | 19.22 |
| CS2 | 78.83 | 18.72 |
| CS3 | 76.60 | 19.85 |
| CS4 | 75.57 | 20.37 |
| K | 80.00 | 18.12 |
| EH | 125.55 | trace |

$\%\mathrm{NaCl} = 0.5884 - 0.005090 \times (\text{mv reading})$

found to have 6.76% Total sugar contents of canola sauce and commercial soy sauce were found to be 8 - 15% and 7.7% respectively (Table 12). Since lactic acid bacteria converts simple sugars into organic acids resulting in a smaller amount of sugars and an increase in acidity in the mash as fermentation proceeds, the difference in sugar contents of the canola and soy sauce might be due to the difference in the length of time employe for the fermentation (5 wk. vs 1 year). Nevertheless, these results demonstrated a remarkable similarity between chemical composition of the canola sauce and the commercial soy sauce.

It was worth noting that the Munson and Walker sugar analysis method employed in this experiment was not very accurate, especially in the samples with minute concentration. A reliability test was therefore performed using pure glucose and sucrose solutions with concentrations similar to that present in the sauce. A deviation of 15% was found among 4 replicates. These results might annotate the slight discrepancy found between the results obtained in this experiment and those reported in literature (Yokotsuka, 1981). Besides the inadequacy of the analytical method used, erroneous results could also be made by the presence of other reducing substances.

Sugar profile of the sauce was obtained with an HPLC. Unfortunately, separation of individual sugars was unsuccessfully carried out. Sarkar et al. (1979) however analyzed their canola sauce produced by acid hydrolysis

Table12. Sugar contents of the canola sauces and the commercial soy sauce

| | % reducing sugar as glucose | % non-reducing sugar as sucrose | % total sugar |
|---|---|---|---|
| CS1 | 8.81 | 6.58 | 15.39 |
| CS2 | 7.43 | 1.26 | 8.69 |
| CS3 | 8.77 | 2.08 | 10.85 |
| CS4 | 9.67 | 0.49 | 10.16 |
| K | 6.76 | 0.95 | 7.71 |
| EH | trace | trace | trace |

and found that major sugars were glucose, fructose and galacturonic acid with a minute amount of maltose, sucrose, xylose and arabinose.

### 4.4.5 Color

Results of color measurement of canola sauce, enzyme hydrolyzate and commercial soy sauce are recorded in Table 13. Kikkoman shoyu with the 'a' value of 8.8 as compared with 7.1, 6.0, 6.4, 6.9 of samples CS1, CS2, CS3, CS4, respectively, appeared to be more reddish , while the 'b' values of 3.0, 2.7, 3.9, 4.6 in CS1, CS2, CS3, CS4 as compared to 3.7 in Kikkoman shoyu respectively, indicated insignificant difference in yellow tint among the samples. Samples CS3 and CS4 with the L values of 6.6 and 7.4 were somewhat lighter as ocmpared to CS1 and CS2 with the value of 5.0.

Since color of the sauce is mainly due to the oxidation of Amadori compounds and their intermediates formed through the reaction between amino acids and sugars, different conditons, especially temperature and time employed in production and pasteurization of the commercial soy sauce and the sauce produced in this experiment might explain the difference in color among the samples. Perhaps, the light reddish color of Kikkoman soy sauce was due to more favorable fermentation conditions during the mash aging (Okuhara and Saito, 1970c). Furthermore, different constituents in raw materials would certainly affect the

Table 13 Color measurements of the canola sauces and the commercial sauce

| | L | a | b |
|---|---|---|---|
| CS1 | 5.0 | 7.1 | 3.0 |
| CS2 | 5.0 | 6.0 | 2.7 |
| CS3 | 6.6 | 6.4 | 3.9 |
| CS4 | 7.4 | 6.9 | 4.6 |
| K | 5.9 | 8.8 | 3.7 |
| EH | 6.8 | 6.3 | 3.8 |

colorization of the sauce as suggested by Hashiba et al. (1981).

### 4.5 Sensory Evaluation

Three sessions of sensory evaluation were conducted using either Kikkoman shoyu, Chinese soy sauce or chemical soy sauce as a control in each session. Aroma, taste and overall acceptance of the samples were rated according to individual preference.

Results obtained from the analysis of variance and Duncan Multiple Range test on the scores from the first session indicated that Kikkoman shoyu was superior in all the three attributes to the canola sauce samoles (Table 14). The averge scores of overall acceptance for the canola sauce (CS1, CS2, CS3, CS4) and Kikkoman shoyu were 4.36, 4.59, 4.55 5.50 and 7.59 respectively. From the general comments of the panelists, Kikkoman shoyu was scored the highest because if its stronger aroma and sweet caramel taste. The canola sauce, on the other hand, was found to have some raw smell and taste. The unpleasant smell in the canola sauce samples might be due mainly to insufficient pasteurization.

Comparison of the canola sauce and Chinese soy sauce in session 2 indicated that the panelists were unable to distinguish any difference in taste among the samples. (Table 15). However, aroma of the canola sauce was still rated inferior to that of the Chinese soy sauce. The rank order of the aroma scores on the samples was Chinese soy

Table 14. Average scores of sensory evaluation on canola sauce and Kikkoman soy sauce.

| | CS1 | CS2 | CS3 | CS4 | K |
|---|---|---|---|---|---|
| Taste | 4.60 | 4.45 | 4.73 | 5.45 | 7.23 |
| Aroma | 4.00 | 4.36 | 4.55 | 4.18 | 6.86 |
| Overall-acceptance | 4.36 | 4.59 | 4.55 | 5.50 | 7.59 |

Table 15. Average scores of sensory evaluation on canola sauce and Chinese soy sauce.

| | CS1 | CS2 | CS3 | CS4 | K |
|---|---|---|---|---|---|
| Taste | 5.50 | 4.80 | 5.20 | 6.20 | 6.70 |
| Aroma | 3.60 | 3.40 | 3.60 | 4.10 | 6.80 |
| Overall-acceptance | 5.05 | 4.55 | 4.95 | 6.00 | 6.80 |

sauce, CS4, CS1, CS3 and CS2. Duncan's Multiple Range test of the results on overall acceptance of the samples revealed that sample CS4 was rated comparable to the Chinese soy sauce, while the remaining samples were rated inferior. The average acceptance scores for the Chinese soy sauce, CS1, CS2, CS3, CS4 were 5.1, 4.6, 4.9, 6.0 and 6.8 respectively.

Results obtained from session 3 using a chemical soy sauce as control were found to be similar to those obtained from session 1. (Table 16). However, sample CS4, which was rated as the second best in all the three organoleptic characteristics in session 1 and 2, was ranked the poorest in this session. This suggested the inconsistancy of the panelists' assessment among the different sessions.

To investigate the reliability of the panelists' assessment, analysis of variance was performed on panelist and sessions. Results indicated that there was a high significantly discrepancy in the judgement among the panelists. Evaluation of the same samples by the same panelists also varied significantly from one session to another. This inconsistency in judgement made the results of sensory assessment questionable. A conclusion, might be drawn, therefore, that the acceptability of a sauce product depended largely on individual preference which varied widely, at least among the panelists used in this investigation. Examination of the assessors revealed that individual preference was greatly influenced by the difference in racial and cultural backgrounds. For example,

Table 16. Average scores of sensory evaluation on canola sauce and China Lily.

| | CS1 | CS2 | CS3 | CS4 | K |
|---|---|---|---|---|---|
| Taste | 5.00 | 4.70 | 4.70 | 3.90 | 6.90 |
| Aroma | 4.90 | 4.40 | 4.30 | 3.20 | 6.90 |
| Overall-acceptance | 4.95 | 4.80 | 4.60 | 3.80 | 7.00 |

canola sauces was more readily accepted by the Orientals than by the Caucasians, as the latter, by and large, were more conditioned to Japanese style soy sauce which is more readily available in Canadian super markets.

In general, canola sauce and soy sauce were comparable in taste to that of commercial soy sauces. However, the former lacked the reminescence of the myriad aroma associated with soy sauce. Several interpretations might be made. Firstly, the canola sauce produced in this research was not fortified with any artificial flavoring agents. On the other hand, monosodium glutamate, alcohol, molasses or caramel were added to most of the commercial soy sauce. These artifical flavors might exert a synergistic effect on the natural taste and aroma of the sauce, making it superior to the raw taste of the canola sauce. Secondly, as concluded earlier the canola sauce was deficient in acid as well as alcohol fermentation which served an important role in producing characteristic aroma and taste in the product. Thirdly, inadequate pasteurization of canola sauce might also result in the failure to bring out desirable aroma and taste in the sauce. Further investigations are, therefore, neede to bring about improvement in organoleptic quality of canola sauce.

# 5. CONCLUSIONS

The feasibility of using canola meal as a raw material substitute for the productin of soy sauce type product has further been confirmed. Instead of the corrosive acid hydrolysis semichemical approach attempted by Ooraikul et al. (1980), a more moderate, yet efficient method employing enzyme to prehydrolyze the canola meal and hasten the subsequent fermentation was demonstrated. Since no addition or modification of equipment already used in the industry was required, application of this process in large scale operation appeared to be practicable. Preliminary work on the enzyme also demonstrated the usefulness of Response Surface Methodology in industrial optimization.

Chemical composition of the sauce produced by this method revealed that the product had superior total soluble solids, amino nitrogen and total soluble nitrogen contents, which denoted the potent proteolytic conversion of the enzyme. Moreover, the high amino nitrogen to total soluble nitrogen ratio indicated that the process had the potential for producing superior product. The overall amino acid content of the canola sauce was slightly lower than that of the commercial soy sauce. However, the sauce had a substantial amount of glutamic acid which was considered to be an essence in flavoring (Udo, 1931). Reducing sugar and salt composition were found to be well within the commercial soy sauce standards.

The greatest deficiencies in the quality of the canola sauce were their low acidity and lack of aroma. These were attributed to the inadequacy of acid and alcohol fermentation. Artificial inoculation of lactic bacteria and yeast would be necessary to bring it to the high standard of Kikkoman shoyu. In spite of these deficiencies, absence of levulinic acid in the sauces suggested that the undesirable compound produced in the acid hydrolysis was not produced in the enzymatic process.

Inconsistency of the judgement by the panels rendered the results of sensory evaluation of the products unreliable. However, general comments by the judges pointed to the fact that the canola sauce possessed some 'raw' smell and did not have the traditional soy sauce aroma. Improvement of the flavor bouquet of the product would require further investigation. Nevertheless, the chemical composition of the canola sauce produced by this method suggested that the products had a great potential to be improved and developed into a distinctive sauce with quality comparable to that of first class soy sauce.

## 6. SUGGESTIONS FOR FURTHER RESEARCH

In order to rectify the deficiencies in quality of canola sauce, further research is necessary. This may include the following :

1. Previous studies indicated that protein digestibiltiy increased when raw materials were soaked and autoclaved. Applying these treatments to canola meal may increase amino acid content in canola sauce.
2. Since inadequate acid fermentation was generally concluded from this study, inoculation of lactic acid bacteria at the beginning of moromi fermentation may help to improve the acid fermentation. Recording bacterial counts during the course of the fermentation may also help to disclose the extent and progress of the fermentation.
3. Absence of alcohol fermentation in the moromi was suspected to be responsible for lack of characteristic aroma in the sauce. Inoculation of yeast to the mash at pH 4.5, achieved either by addition of lactic acid, or lactic acid bacteria, is anticipated to ameliorate the flavor of the sauce.
4. Pasteurization at higher temperature for a longer time may help get rid of the raw taste as well as darken the color of the sauce.
5. Sauce quality may also be improved through fortification with artificial flavors or addition of suitable enzymes at suitable stages of fermentation. This would, of

course, involve extensive study of biochemistry of sauce fermentation.

6. Finally, trained panelists were recommended for sauce quality evaluation so that a more reliable judgement may be obtained.

# 7. BIBLIOGRAPHY

Aidoo, K.E., Hendry, R. and Wood, B.J.B. 1981. Amyloglucosidase and maltase activities in soy sauce fermentation. J. Fd. Technol. 18:543 - 548

Aishma,Y. 1977a. Objectives specification of food flavor: analysis of gas chromatography by stepwise regression analysis. Fd. Chem. 2(2):81 - 84

Aishma,Y. 1977b. Flavor evaluation by multiple regression analysis of gas chromatographic patterns in soy suace. Fd. Chem. 2(3):161 167

Aishma, T. 1981. Glass capillary chromatographic profiles of soy sauce aroma correlated with sensory assessment. Agric. Biol. Chem. 45(12): 2847 - 2853.

A.O.A.C. 1970. Improved Kjeldahl method for nitrate-free samples (18) - official final action. Section 2.051

A.O.A.C. 1975. Sugars and sugar products. Section 31.026, 31.038, 31.041, 52.018

Asao, Y., Sakasai, T. and Yokotsuka, T. 1967. Role of yeasts in the production of shoyu (soy sauce) flavor. J. Agric. Chem. Soc. Jap. 41:434 - 441

Asao, Y. and Yokotsuka, T. 1957a. Studies on flavorous substances in soy sauce. XVI. Flavorous ingredients in raw soy sauce. ( 2 ) Acidic compounds. Bull Agric. Chem. Soc., Japan. 32:617 - 622.

Asao, Y. and Yokotsuka, T. 1957b. Studies on flavorous substances in soy sauce. XVII. ( 1 )Guaiacyl compounds in soy sauce. Bull. Agric. Chem. Soc. Japan. 32:622 - 628.

Baens-Arcega, L. 1966. Sauce manufacture. Process Biochemistry 5(10):50 - 56

Box, G.E.P. and K.B. Wilson 1951. On the experimental attainment of optimum conditions (with discussion). J.R. Statist. Soc. 313:1

Campbell,L.D. and Eggum,B.O. 1980. Protein Quality of Canola Meal. Research on Canola Seed. Oil, Meal and Meal Fraction - 6th progress report. Canola Council of Canada. Publication no.57. p.243 - 245

Camirand, W., Randall, J., Popper, K and AAndich, B. 1983. Low-sodium/high potassium fermented sauces. Fd. Technol. 35:81 - 84

Church,M.B. 1923. Soy and Related Fermentation. Spice Mill 47:168 - 173

Constantinidines, A. and Adu, A. 1980. Enzymatic modification of vegetable protein: mechanism, kinetics and production of soluble and partially soluble protein in a batch reactor. Biotechnology and Bioengineering 22:543 - 565

Delory, G.E. and E.J. King 1945. A sodium carbonate-bicarbonate buffer for alkaline phosphatase. Biochem. J. 39:245

Eriksen, S. 1983. Application of Enzymes in Soy Milk Production to Improve Yield. J. Fd. Sc. 48:445 - 447

Freedman, D., Pisani, R., and Purves, R. 1978. Statistics (1st ed.). W.W. Norton and Company. N.Y., p.109 - 186

Fukushima, D. 1979. Fermented vegetable (soybean) protein and related foods of Japan and China. J.A.O.C.S. 56(1):357 - 362

Fukushima, D. 1981. Soy proteins for foods centering around soy sauce and tofu. J.A.O.C.S. 58:346 - 354

Goel, S.K. and Wood B.J.B. 1978. Technical note: cellulase and exo-amylase in experimental soy sauce fermentation. J. Fd. Technol. 13:243 - 247

Groff, E.H. 1919. Soy-sauce manufacturing in Kwangtung, China. Phillipines J. of Science 15:307 - 315

Hamada, S., Gamo, J., Kadosawa, T., and Aso, K. 1956. On the sugar compounds in soy sauce. J. Fermentation Technol. 34:407 - 410

Harada,Y. and Toyama, N. 1966. Applications of cellulase in brewing . IV. Isolation of soybean protein with cellulase and a cell-separating enzyme. 44(11):835 - 841

Hashiba, H. 1976. Participation of Amadori rearrangement products and carbonyl compounds in oxygen-dependent browning of soy sauce. J. Agric. Food Chem. 24(1):70 - 74

Hashiba, H. 1978. Isolation and identification of Amadori compounds from soy sauce. Agric. Biol. Chem. 42(4):763 - 768

Hashiba, H., Okuhara, A. and Iguhchi, A. 1981. Oxygen-dependent browning of soy sauce and some brewed products. Prog. Fd. Nutr. Sci. 5(1):93 - 113

Hesseltine, C.W. and Wang, H.C. 1979. Fermented soybean food products. In: Soybeans - Chemistry and Technology vol.1 - Proteins. Smith, A.K. ed. AVI Publishing Company, Inc., Westport, Conneticut. p.397 - 401

Impoolsup, A., Bhumiratana, A. and Flegel, T.W. 1981. Isolation of alkaline and neutral proteases from *Aspergillus flavus* var. columnaris, a soy sauce koji mold. Applied and Environmental Microbiology 42(2):619 - 628

Ishimaru, Y. 1933. Bacteria associated with the manufacture of soy. J. Agr. Chem. Soc. Jap. 9:859 - 904 and 953 - 1004

Kanbe, C. and Uchida, K. 1982. Diversity in the metabolism of organic acids by Pediococcus halophilus. Agric. Biol. Chem. 46(9):2357 - 2359

Kandachi, M. and Nishi, H. 1949. On the effect of protein precipitants for sugar determination by Betrand's method. J. Agr. Chem. Soc. Japan 23:194 - 199

Kawano, Y. 1938. The ingredient change of soybean cooking. J. Brewing 16:755 - 767

Kato,T and Matsumoto,K 1964. Soybean Hull. 2. Hydrolyzate of soybean hull prepared by using cellulase. Nippon Jozo Kjokai Zasshi 59(5):431 - 434

Kihara, K. 1938. The comparison between fermented soy sauce and hydrochloric acid hydrolyzate of soybean. J. Brewing 16:919 - 936

Kundu, A.K. and Manna, S. 1975. Purification and characterization of extracellular proteinases of *A. oryzae*. Applied Microbiolgy 30(4):507 - 513

Kuninaka, A., Rokugawa, K., and Yoshino, H. 1980. Conidia of *Aspergillus oryzae* naturally immobilized phosphatases. Agric. Biol. Chem. 44(12):1825 - 2829

Lacroix et al. 1983. Hydrolyse enzymatique des proteines de colza. Can. Inst. Food Sci. Technol. J. 16(4):242 - 245

Lockwood, L.B. 1947. The production of Chinese soya sauce. Soybean Digest 7(12):10 - 12

Lodder, J. and Kreger-van Rij, N.J.w. 1952. The Yeast. A taxonomic study. North - Holland Pub., Amsterdam.

Lukas,A.J. 1971a Fermenting whey and producing soy sauce therefrom U.S. Patent 3552981

Lukas,A.J. 1971b Fermentating whey for producing soy sauce. U.S. Patent 3558328

Matsumoto, K. 1925. Change of buffer action upon the saccharization of rice malt. Rep. Gov't. Inst. Brew. Japan 99:1 - 187

Montgomery, D.C. 1984. Response surface methodology. In: Design and Analysis of Experiments (2nd ed.). John Wiley and Sons. N.Y.

Minor, L.J. 1945. Soya sauce processes and how they can be improved. Food Industries 17:758 - 760

Mori, S. 1979. Qualty evaluation of shoyu and application off factor analysis. J. Brew. Soc. Japan. 74(8):526 - 531

Myers, R.H. 1971. Response Surface Methodology. Allyn and Bacon Inc. Boston

Nakadai, T. and Nasuno, S. 1976. Enzymatic hydrolysis of protein by various enzyme preparations. J. Ferment. Technol. 54(12):872 - 884

Noda, F., Hayashi, K., and Mizunuma, T. 1980. Antagonism between osmophylic lactic acid bacteria and yeasts in brine fermentation of soy sauce. Appl. and Environ. Microb. 40(3):452 - 457

Noda, F., Hayashi, K., and Mizunuma, T. 1982. Influence of pH on inhibitory activity of acetic acid on osmophylic yeasts used in brine fermentation of soy sauce. Appl. and Environ. Microb. 43(1): 245 - 246

Noda Soy Sauce Co., Ltd. 1955. The treatment of soybean in soy sauce fermentation. Noda-shi, Chiba-ken, Japan

Novo Industri A/S. Alcalase 0.6L. Technical Data.

Novo Industri A/S, Enzyme Division. Novo Alle, DK-2880, Dagsvaerd, Denmark

Novo Industri A/S. Enzymes at Work. Novo Alle, DK-2880 Bagsvaerd, Denmark

Novo Industri A/S. Enzyme Information Sheet. Proteolytic enzymes for the modification of food proteins. Description and application of alcalase 0.6L and neutrase 0.5L

Nunomura, ~~N.,~~ Sasaki, M., and Asao, Y. 1976a. Identification of volatile components in shoyu (soy sauce) by gas chromatography mass spectrometry. Agric. Biol. Chem. 40(3):485 - 490

Nunomura, N., Saskai, M. and Asao, Y. 1976b. Isolation and identification of 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone one, as a flavor component. Agri. Biol. Chem. 40(30:491 - 495

Nunomura, N., Sasaki, M., and Asao, Y. 1978. Shoyu (soy sauce) volatile flavor components: basic formation. Agric. Biol. Chem. 42(11):2123 - 2128

Nunomura, N., Sasaki, M., and Yokotsuka, T. 1979. Isolation of 4-hydroxy-5-methyl-3(2H) furanone, a flavor component in shoyu (soy sauce). Agric. Biol. Chem. 43(6): 1362 - 1363

Nunomura, N., Sasaki, M., and Yokotsuka, T. 1980. Shoyu (soy sauce) flavor components: acidic fractions and the characteristic flavor component. Agric. Biol. Chem. 44(2):339 - 351

Oba, T. 1974. Browning of rice-koji. VI. Comparison of characteristics of tyrosinase extracted from rice-koji with those of tyrosinase extracted from the mycellium. Nippon Jazo Kyokai Zasshi 69:56

Oda, M., Ikeda, K. and Tanimoto, M. 1949. Aspergillus fungi which produce the most effective tannase and the application of thes fungi to the production of substitute soy sauce. J. Fermentattion Technol. 27:16

Ohara, H. and Moriguchi, S. 1955. Soy sauce fermentation under various conditions in the preparation of mash. II. On the ratio of carbon and nitrogen. Seasoning Sci. 3(3):18 - 26

Okuhara, A., Saito, N. and Yokotsuka, T. 1971. The color of soy sauce. VI. The effect of peptides on browning. J. Fermentation Technol. 27:16 - 23

Okuhara, A. and Saito, N. 1970a. Color of soy sauce. III. Effect of reducing reagents on browning of soy sauce. Ibid. 48(3):177 - 189

Okuhara, and Saito N. 1970b. Color of soy sauce. V. Application of multiple correlation analysis tto analysis of browning mechanism. Ibid. 48(4):228 - 236

Okuhara, A. and Saito, N. 1970c. Color of soy sauce. IV. Decolorization and depression of browning by reduction. Ibid. 48(3):190 - 201

Olsen, H.S. and Nissen, J.A. 1979. Industrial production and applications of a soluble enzymatic hydrolyzate of soya protein. Process Biochemistry p.6 - 11

Onaga, D.M., Luh, B.S., and Leonard, S.J. 1957. Quality evaluation and chemical composition of soy sauce. Food Research 22:83 - 88

Onishi, H. 1957. Studies on osmophilic yeasts. II. Factors affecting growth of soy yeasts and others in the environment of a high concentration of sodium chloride. Bull. Agric. Chem. Soc. Japan 21:143 - 150

Onishi, H. and Saito, N. 1961. Osmophilic yeasts. XII. Characteristic aspects on the assimilation and fermentation of sugars by osmophilic yeastts in the environment of a high concentraation of sodium chloride. Agr. Biol. Chem. 25(5):341 - 349

Ohloff, G. Schulte-Elte, K.H., Gauttschim, F., Renold, W. 1978. Vitispiranes, important constituents of vanilla aroma. Helvetica Chimica Acta. 61(3):1125 - 1133

Ooraikul, B., Mei, H.M., Sarkar, S.K. and Jackson, H. 1980. J. Fd. Sc. 45:200 - 203.

Pearson, D. 1976. The Chemical Analysis of Foods.( 7th, ed.) Churchill Livingstone, New York. PP. 9 - 11

Piper,C.V. and Morse,W.J. 1943. The Soybean. Peter Smith. N.Y.

Reddy et al. 1982. Legume - based fermented foods: their preparation and nutritional quality. CRC critical review in Food Science and Nutrition. 17(4):335 - 370.

Sakaguchi, K. 1958. The activities of bacteria in soybean sauce brewing. II. The proteinases and the occurrence of bacterial spores in the soy mash. Bull. Agr. Chem. Soc. Japan. 22:345 - 352.

Saskasai, T. and Yokotsuka, 1957. T. Noda soy sauce Co. Ltd. Unpublished data.

Sarkar, S.K., Mei, H.M. and Ooraikul, B. 1979. Unpublished data. Dept. of Food Science. Univ. of Alberta. Alta.

Sasaki, M. and Nunomura, N. 1981. Studies of flavor constituents of soy sacue. VII. Constituents of topnote aroma of soy sauce. Nippon Kagaku Kaishi (Japan) 5:736 - 745

Sekine, H. 1976. Netrual proteinases I and II of *Aspergillus sojae* action on various substrates. Agr. Biol. Chem.

40(4):703 - 709.

Singh,D. 1958. Rape and Mustard, Indian Central Oilseeds committee, Bombay.

Smiley, K.L., Hensley, D.E., and Gasdorf, H.J. 1976. Alpha-galactosidase production and use in a hollow-fiber reactor. Applied and Environmental Microbiology 31(4):615 - 617

Smith, A. K. and Circle, S. J. 1972. Fermented soybean food products. In: Soybean - chemistry and technology. p.397 - 401

Smith J. 1982. Ph.D. Thesis: Department of Food Science, University of Alberta

Somogyi, M. 1952. Notes on sugar determination. J. Biol. Chem. 195:19 - 23

Sorensen, S.P.L. 1909. Supplement to the Paper. Enzyme studies (II) concerning the measurement and significance of the hydrogen ion concentration in enzymatic process. Biochem. Z. 22:352 - 356

Sosulski, F.W. and Sarwar, G. 1973. Amino acid composition of oil seed meals and protein isolates. Can. Inst. Sci. Technol. J. 6(1):1 - 5

Sprossler, B. and Plainer, H. 1983. Immobilized lactase for processing whey. J. Fd. Technol. 18:93 - 98

Stanley, D.W. 1981. Non-bitter protein hydrolysate. Can. Inst. Food Sci. Technol. J. 14(1):49 - 52

Steinkraus, K.H. 1983. Chinese soy sauce, Japanese shoyu, Japanese miso, Southeast Asian fish sauces and pastes, and related fermented foods: Indigenous amino acid/peptide sauces and pastes with meat like floavors. In: Handbook of Indigenous Fermented Foods. Microbiology Series, vol.9. Marcel Dekker, Inc. N.Y. p.433 - 467

Steel, R.G.D. and Torrie, J. 1980. Principles and Procedures of Statistics - A Biometrical Approach (2nd ed.). McGraw-Hill Book Company N.Y.

Summers,J.D. 1973. Research on rapeseed oil and meal - 2nd progress repor. Publication no.32 p.76

A Synopsis of Japanese Traditional Fermented Foodstuffs. Reference for the final topic session - 13 ( Mashahiro, editor). 4th International Fermentation Symposium 19-25 March, 1972. Kyoto. Japan.

Takahashi, T. and Yukawa, M. 1911. The budding fungi of shoyu - moromi. J. Agr. Soc. Jap. 112:1 - 26.

Takahashi, J. and Yukawa, M. 1914. The budding fungi of 'shoju-moromi'. Orig. Com. 8th Inter, Appl. Chem. 14:155 - 71

Tamura, J. and Aiba, T. 1982. Purifiction and some properties of N - material in soy sauce. Agric. Biol. Chem. 46(5):1291 - 1300.

Tsujita, Y. and Endo, A. 1976. Purification and characterization of the two molecular forms of *Aspergillus oryzae* acid proteases Biochemica et Biophysica Acta. 445:194 - 204.

Tsukakara, T. 1948. Ultilizing the garbage from the allied forces. Hakko Kyokaishi. 6:49

Tsunoda, T. and Ishizuka, Z. 1952. Amino acids composition of fermented soy sauce determined by bioassay. J. Agri. Chem. Soc. Japan. 26:477 - 480.

Ueda, R., Nagai, S., Kambara, K. and Moriguchi, S. 1958. Studies on the organic acids in soy sauce ( I ) detn., ( II ). J. Fermentation Technol. 37:94 - 104.

Umeda, I., Nakamura, K., Yamato, M. and Nakamura, Y. 1969. Investigation of comparative production of shoyu (soy-sauce) from deffated soybean meals obtained from United States and Japanese soybeans and processed by United States and Japanese methods. USDA Final Tech. Rept. Public Law 480. Project UR-ALL-(40)-21

Udo, S. 1931. Studies on the taste of soy sauce. II. On the organic bases in soy sauce. J. Agr. Chem. Soc. Japan. 7: 852 858.

Watanable, H. 1984. Traditional miso and soy sauce remain favorites of Japanese. Bussiness Japan. 49:

Watanabe, T. 1969. Industrial production of soybean foods in Japan. In: UNIDO Expert Group Meeting on Soybean Processing and Use, USDA. Peoria III. Nov.,17 - 21 ( Proc. in Press ).

Wood, B.J. 1982. Soy sauce and miso. In: Economic Microbiology ( Rose, A.H. ed. ) 7: 39 - 86. Academic Press. N.Y.

Yokotsuka, T. 1960. Aroma and Flavor of Japanese soy sauce. Adv. in Fd. Res. 10:75 - 133.

Yokotsuka, T. 1977. Japanese shoyu. Symposium on Indigenous Fermented Foods. Bangkok, Thailand.

Yokotsuka, T. 1981. Recent advances in shoyu research. In: The Quality of Food and Beverages. Academic Press. Inc., p.171 - 196.

Yokotsuka, T., Asao, Y. and Saskasai, T. 1967b. Studies on flavorous substances in shoyu part 27. The production of 4-ethyl-guaiacol during shoyu fermentation and its role for shoyu flavor. J. Agric. Chem. Soc. Japan. 41:442 - 447.

Yokotsuka, T., Takimoto, k. and Izuka, S.. 1955. Studies on the analytical method of total nitrogen in soy sauce by Kjeldahl method. Seasoning Sci. 13:9 - 16

Yokotsuka, T., Sakasai, T. and Asoa, Y. 1967a. Studies on flavorous substances in shoyu. Part 25. Flavorous compounds produced by yeast fermentation. J. Agric. Chem. Soc. Japan. 41: 428 - 433.

Yong, F. M. 1971. M.Sc. Thesis. University of Strathclyde, Glasgow.

Yong, F.M. and Lee, K.H. and Wong, H.A.. 1978. Study of factora affecting the growth of soy yeast *Saccharomyces rouxii* NRRLY - 1096. J. Fd. Technol. 13:385 - 396.

Yong, F.M., Lee, K.H. and Wong, H.A.. 1980. The production of volatile acids from glucose by soy yeast ( *Saccharomyces rouxii* ) NRRLY - 1096. J. Fd. Technol. 15:421 - 428.

Yong, F.M., Lee, K.H. and Wong, H.A.. 1981. The production of ethyl acetate by soy yeast *Saccharomyces rouxii* NRRLY - 1096. 16(2):177 - 184.

Yong, F.M. and Wood, B,J.B.. 1974. Microbiology and biochemistry of soy sauce fermentation. Adv. Appl. Microbiol. 17:157 - 230.

Yong, F.M. and Wood, B.J.B. 1976 Microbial succession in experimental soy sauce fermentation. J. Fd. Technol. 11:525 - 536

Yong, F.M. and Wood, B.J.B.. 1977a. Biochemical changes in experimental soy sauce koji. J. Fd. Technol. 12:163 - 175

Yong,F.M. and Wood,B.J.B. 1977b. Biochemical changes in experimental soy sauce moromi. J. Fd. Technol. 12:263-273.

Yoshida, M, Kuriharaa, E. and Yamanishi, T. 1980. Topnote aroma components of soy sauce. Eiyo to Shokuryo. 33(1):39 - 40